PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

BB

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12N 9/16, C07K 7/08
A61K 37/02, 39/392, C12N 15/00

A1

(11) International Publication Number: WO 89/09818

(43) International Publication Date: 19 October 1989 (19.10.89)

(21) International Application Number: PCT/US89/01418

(22) International Filing Date: 11 April 1989 (11.04.89)

(30) Priority data:

181,893	15 April 1988 (15.04.88)	US
219,491	12 July 1988 (12.07.88)	US

(71) Applicant: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors: KRAMER, Ruth, M. ; 3740 Governor's Road, Golden Hill, IN 46208 (US). PEPINSKY, R., Blake ; 69 Parker Street, Watertown, MA 02172 (US). HESSION, Catherine ; 96 Fountain Lane, South Weymouth, MA 02190 (US).

(74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 875 Third Avenue, New York, NY 10022-6250 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROCESSES FOR PURIFYING PHOSPHOLIPASE A_2 AND PRODUCING PHOSPHOLIPASE A_2-LIKE POLYPEPTIDES

(57) Abstract

This invention relates to processes for purifying acid stable phospholipase A_2. Specifically, the invention relates to processes for the purification of phospholipase A_2 form biological sources, such as non-pancreatic human sources which contain very small amounts of that enzyme. More specifically, this invention relates to the purification and characterization of phospholipase A_2 from human platelets and from human rheumatoid synovial fluid. This invention also relates to polypeptides corresponding to at least a portion of the amino terminal amino acid sequence of human platelet and rheumatoid synovial fluid phospholipase A_2 and antibodies thereto, as well as antibodies to purified, intact acid-stable phospholipase A_2 for use in the treatment or diagnosis of inflammation and tissue injury associated with various diseases. And this invention relates to DNA sequences which encode these polypeptides. This invention further relates to methods for producing phospholipase A_2 in hosts transformed with recombinant DNA molecules comprising those DNA sequences.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

PROCESSES FOR PURIFYING PHOSPHOLIPASE A_2 AND PRODUCING PHOSPHOLIPASE A_2-LIKE POLYPEPTIDES

TECHNICAL FIELD OF INVENTION

This invention relates to processes for purifying acid stable phospholipase A_2. Specifically, the invention relates to processes for the purification of phospholipase A_2 from biological sources, such as non-pancreatic human sources which contain very small amounts of that enzyme. More specifically, this invention relates to the purification and characterization of phospholipase A_2 from human platelets and from human rheumatoid synovial fluid. This invention also relates to polypeptides corresponding to at least a portion of the amino terminal amino acid sequence of human platelet and rheumatoid synovial fluid phospholipase A_2, and antibodies thereto, as well as antibodies to purified, intact, acid-stable phospholipase A_2 for use in the treatment or diagnosis of inflammation and tissue injury associated with various diseases. This invention further relates to DNA sequences which encode human inflammatory phospholipase A_2 and methods for producing phospholipase A_2 in hosts transformed with

recombinant DNA molecules comprising those DNA sequences.

BACKGROUND ART

Phospholipases A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4, PLA_2) are a family of lipolytic proteins that specifically cleave the acyl ester linkage at the sn-2 position of glycerophospholipids. These enzymes are ubiquitous and are present in virtually every cell type from bacteria to man. Nearly all of the phospholipases A_2 studied to date have a molecular weight of between 10 and 15 kilodaltons, but they differ substantially in amino acid sequence. Secretory phospholipases A_2 may be divided into two categories: digestive (produced and secreted by digestive organs, such as the pancreas), and inflammatory (produced and secreted by inflammatory cells, such as platelets or neutrophils, or found in inflammatory fluids, such as rheumatoid synovial fluid).

In mammals, phospholipase A_2 is found in abundant quantities in the pancreas. Other cellular and extracellular mammalian phospholipases A_2 are found in much smaller amounts. In humans, non-pancreatic phospholipases A_2 have been found in seminal plasma, synovial fluid, septic shock serum, and bronchoalveolar lavage fluid of alveolar proteinosis (P. Vadas and W. Pruzanski, "Biology of Disease. Role of Secretory Phospholipases A_2 in the Pathobiology of Disease", Lab. Invest., 55, pp. 391-404 (1986)). Most of the mammalian phospholipases A_2 are acid-stable and all are are calcium-dependent to varying degrees. To date, with one exception, non-pancreatic phospholipases A_2 demonstrate no immunological cross-reactivity with pancreatic phospholipase A_2 (J. G. N. DeJung et al. "Monoclonal Antibodies Against an Intracellular Phospholipase

A_2 from Rat Liver and their Cross-Reactivity with Other Phospholipases A_2", Eur. J. Biochem., 164, pp. 129-35 (1987)).

Intracellular phospholipases A_2 are involved in various physiological functions, including membrane phospholipid turnover, repair of membrane peroxidation damage, transmembrane signaling, cell membrane dynamics and generation of lipid mediators. The control and regulation of phospholipases A_2 is complex and involves many factors, including free calcium concentration, molecular entities involved in transmembrane signaling, and the physiochemical state of the phospholipid substrate (H. van den Bosch in Comprehensive Biochemistry, vol. 4, pp. 313-57, J. N. Hawthorne and G. B. Ansell, eds., Elsevier Amsterdam (1982)).

Phospholipases A_2 are also known to mediate a variety of pathophysiological conditions through the products of protein catalysis -- lysophospholipids and arachidonic acid (J. Chang et al., "Phospholipase A_2: Function and Pharmacological Regulation", Biochem. Pharmacol., 36, pp. 2429-36 (1987); P. Vadas and W. Pruzanski, Lab. Invest., 55, pp. 391-404 (1986); A. A. Farooqui et al., "Phospholipases, Lysophospholipases, and Lipases and Their Involvement in Various Diseases", Neurochem. Path., 7, pp. 99-128 (1987)). Lysophospholipids are fusogenic and cytotoxic. Subsequent metabolism of phospholipase A_2 catalytic products by certain protein cascades leads to several potent, biologically active substances. These include prostaglandins, hydroxylated fatty acids, leukotrienes and platelet activating factor, all of which have been implicated in inflammation or hypersensitivity, or both. Many studies have indicated that phospholipases A_2 play important roles in inflammation and tissue injury associated with various diseases, such as viral and bacterial infections,

skin and connective tissue diseases, such as psoriasis, gastrointestinal disorders, such as pancreatitis and ulcers, ischemias, myocardial infarction, atherosclerosis, pulmonary dysfunctions, such as asthma, acute respiratory distress syndrome and alveolar proteinosis, septic shock, thrombosis, multiple sclerosis, demyelinating diseases and rheumatoid arthritis. Thus, inhibition or inactivation of pathogenic phospholipase A_2 activity is of clinical importance.

Pancreas-derived phospholipase A_2 has been purified, sequenced and structurally defined (H. M. Verheij et al., "Structure and Function of Phospholipase A_2", Rev. Physiol. Biochem. Pharmacol. , 91, pp. 91-203 (1981)). The protein is produced in the form of an inactive precursor which is stored in secretory granules. Once secreted in the intestine the precursor is activated by limited tryptic proteolysis, leading to the formation of the active phospholipase and a small polypeptide. No evidence for such a precursor has been obtained with respect to inflammatory phospholipases A_2.

To date, only three mammalian non-pancreatic phospholipases A_2 have been purified to homogeneity as evidenced by internal sequence analysis. These include phospholipase A_2 from rabbit inflammatory peritoneal exudate (S. Forst et al., "Structural and Functional Properties of a Phospholipase A_2 Purified from an Inflammatory Exudate", Biochemistry, 25, pp. 8381-85 (1986)), phospholipase A_2 from secreted rat platelets (M. Hayakawa et al., "Amino Acid Composition and NH_2-Terminal Amino Acid Sequence of Rat Platelet Secretory Phospholipase A_2", J. Biochem., 101, pp. 1311-14 (1987)), and phospholipase A_2 from rat inflammatory peritoneal exudate (H. W. Chang et al., "Purification and Characterization of Extracellular Phospholipase A_2 from Peritoneal Cavity of Caseinate-

Treated Rat", J. Biochem., 102, pp. 147-54 (1987)). Many attempts have been made to isolate phospholipases A_2 from human non-pancreatic sources, but none has succeeded in purifying these proteins to homogeneity. Thus, to date, the primary structure of these phospholipases A_2 has not been identified.

The need exists for a process for purifying acid stable phospholipases A_2 to homogeneity which, advantageously, also permits purification of the protein from human non-pancreatic biological sources.

DISCLOSURE OF THE INVENTION

The present invention solves the problems referred to above by providing processes for purifying acid stable phospholipases A_2 from biological sources, such as mammalian cells and extracellular fluid, plant cells, insect cells, yeast and other fungi, and bacteria. Specifically, these processes permit the purification of inflammatory phospholipases A_2 to homogeneity from human, non-pancreatic sources such as platelets and rheumatoid synovial fluid. The phospholipases A_2 purified from human platelets and rheumatoid synovial fluid by the processes of this invention are characterized by a common amino-terminal amino acid structure, which differs significantly from that of pancreatic-derived phospholipase A_2. According to one embodiment of this invention, phospholipase A_2 purified from rheumatoid synovial fluid exhibits an inflammatory activity that is at least 100-fold greater than that of the pancreatic-derived protein in an assay of inflammatory action that measures formation of paw edema in the rat after subplant or injection of purified phospholipase A_2 (S. Brain et al, "Action of Phospholipase A on Mast Cell Histamine Release

and Paw Edema in the Rat", Brit. J. Pharmacol., 59, pp. 440-41 (1977)).

This invention also relates to the production of polypeptides corresponding to at least a portion of the amino acid sequence of these inflammatory phospholipases A_2. Such polypeptides, as well as antibodies to these polypeptides and antibodies to intact, purified phospholipases A_2, are useful for pharmacological, therapeutic and diagnostic purposes. In addition, DNA sequences encoding all or part of the deduced amino acid sequence of these phospholipases A_2 are useful as diagnostics for the evaluation and monitoring of diseases, or as probes for the isolation of cDNA or genomic clones coding for human inflammatory phospholipases A_2.

This invention also relates to the production of phospholipase A_2 using recombinant techniques. In such a process, DNA sequences coding for the phospholipases A_2 of this invention, recombinant DNA molecules characterized by those sequences and unicellular hosts transformed with those molecules are employed to produce phospholipases A_2 by culture of those transformed hosts.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the elution profile of a partially purified preparation of human platelet phospholipase A_2 off of a cation exchange chromatography column.

Figure 2 depicts the elution profile of a partially purified preparation of human platelet phospholipase A_2 off of a gel filtration column.

Figure 3, panel A, depicts the elution profile of a partially purified preparation of human platelet phospholipase A_2 off of a reverse phase HPLC column. Figure 3, panel B, depicts the corres-

ponding phospholipase A_2 activity of the eluted fractions.

Figure 4 depicts in tabular form the purification of phospholipase A_2 *from* isolated human platelets.

Figure 5 depicts an Immobilon blot of the human platelet phospholipase A_2-containing fractions collected following HPLC.

Figure 6 depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A_2 off of a cation exchange chromatography column.

Figure 7 depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A_2 off of a gel filtration column.

Figure 8, panel A, depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A_2 off of a reverse phase HPLC column. Figure 8, panel B, depicts the corresponding protein activity of the eluted fractions.

Figure 9 depicts in tabular form the purification of phospholipase A_2 from human rheumatoid synovial fluid

Figure 10 depicts an Immobilon blot of the human rheumatoid synovial fluid phospholipase A_2-containing fractions collected following HPLC.

Figure 11A depicts portions of sequencing plasmid pNN01. Figure 11B depicts the restriction map of PSQ 130.

Figure 12 depicts the DNA sequence of 6.2 kb HindIII fragment of PLA_2 8.5 EMBL3 and the amino acid sequence of human inflammatory phospholipase A_2 derived therefrom.

Figure 13 depicts a comparison of the amino acid sequences of bovine pancreatic phospholipase

A_2, phospholipase A_2 from C. atrox venom, and the derived amino acid sequence from the 6.2 kb HindIII fragment of PLA_2 8.5 EMBL3.

Figure 14A depicts schematically the synthesis of BG368 from BG312. Figure 14B depicts the restriction map of PLA_2 6.2 BG368 3(+). Figure 14C depicts the restriction map of PLA_2 3.8 BG341(+). Figure 14D depicts the restriction map of BG341.

Figure 15A depicts a restriction map of pJODS. Figure 15B depicts a restriction map of PLA_2 3.8 JODS.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to processes for isolating and purifying acid stable phospholipases A_2 from biological sources. According to this invention, phospholipases A_2 may be purified to homogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and amino-terminal amino acid sequence analysis. This invention also relates to polypeptides which are characterized by at least a portion of the amino terminal 19 amino acids of human inflammatory phospholipase A_2 purified from platelets and rheumatoid synovial fluid and to the DNA sequences which encode them.

Generally, one embodiment of the process of this invention begins with the step of acid-extracting the protein from a biological source, such as sonicated platelets or rheumatoid synovial fluid. The acid in the extract is then exchanged for a buffer suitable for cation exchange chromatography and the preparation contacted with a cation exchange resin. The phospholipase A_2 is then eluted from the resin, concentrated and further purified away from dissimilar molecular weight contaminants by molecular sizing. Fractions containing phospholipase A_2 activity are purified to homogeneity by

reverse-phase HPLC. According to another embodiment, the process of this invention further comprises the step of electrophoresing said phospholipase A_2 and transferring said electrophoresed phospholipase A_2 to a solid support.

According to an alternate embodiment of this invention, which permits the isolation of an intracellularly located phospholipase A_2, the process comprises the further initial step of extracting or releasing the protein from the cell. This may be achieved by any of a number of well-known lysing techniques, such as sonication, homogenization, French press, chemical lysis or enzymatic lysis. Mechanical lysis techniques are preferable, because they do not introduce any extraneous proteins or organic chemicals into the phospholipase A_2 preparation. The most preferred method of lysis is sonication.

Acid extraction of the protein according to this invention may be achieved with any acid of any concentration having a pH below about 4.5. Preferably, the acid is a mineral acid, such as hydrochloric acid, phosphoric acid or sulfuric acid and has a pH or about 1.0. The most preferable acid is sulfuric acid at a concentration of about 0.18 N, which gives a pH of about 1.

In order to perform cation exchange chromatography on the phospholipase A_2 preparation, the protein should be in a buffer that is compatible with the cation exchange resin. Numerous methods of achieving buffer exchange are known in the art, including dialysis, ultrafiltration and desalting. Because the phospholipase A_2 is a small protein of about 13,000 daltons, dialysis or ultrafiltration must be carried out using a membrane of suitably small pore size. The most preferred method of buffer exchange is dialysis using a membrane that has a molecular weight cutoff of about 3500 daltons.

Various cation exchange resins that are commercially available may be used in the processes of this invention. Examples of some of these resins are Fast S, Mono S, CM-Sepharose, SP-Sepharose and phosphate-cellulose. The preferred properties of the resin used in the process of this invention are high flow rate, the ability to bind phospholipase A_2 at the cation concentration of initial contact and the ability to release phospholipase A_2 under higher ionic conditions. Cation exchange may be performed batchwise, or preferably in a column. The most preferable conditions for cation exchange are initially binding the protein to a Fast S column in 200 mM NaCl, 50 mM acetate, pH 4.5 and eluting the protein with a linear gradient of 200 mM - 2 M NaCl. Using these conditions, the protein will elute at about 1 M NaCl.

Active fractions from cation exchange may be concentrated by any standard technique, preferably one which does not concurrently concentrate ions. The most preferable means of concentration is ultrafiltration using a membrane with a low molecular weight cutoff, such as a YM 5 membrane (Amicon).

Any one of a number of commercially available molecular sizing chromatography resins may be employed in the processes of this invention. Preferably, the resin will be such that the phospholipase A_2 will elute in the included volume. In this manner, the majority of higher molecular weight contaminants will be removed by elution in the void volume of the column. Among the preferred molecular sizing resins are Biogel P30, Biogel P60, Sephadex G-25, Sephadex G-50, Sephadex G-75 and Utragel AcA54. The most preferred resin is Sephadex G-50 superfine.

Active fractions are further purified by reverse phase HPLC. Any hydrophobic resin that is

compatible with HPLC may be used with the process of this invention. Examples of preferred resins are C18, C8, C4, C3, and phenyl. The most preferred resin is C4.

This invention also relates to phospholipases A_2 produced according to the above described processes. Phospholipase A_2 is most preferably characterized by enzymatic activity. For example, phospholipase A_2 cleaves and releases into the assay supernatant [^{3}H]-oleic acid from autoclaved, [^{3}H]-oleic acid-labelled E.coli. Other phospholipase A_2 substrates include, but are not limited to, phosphatidylcholine and phosphatidylethanolamine.

In addition, various methods of substrate dispersion, such as sonication, solubilizing in organic solvents and mixing with detergents, as well as alternate assay conditions may be employed to characterize phospholipase A_2. All of these assay methods are well-known in the art (L. R. Ballou and W. Y. Cheung, "Marked Increase of Human Platelet Phospholipase A_2 Activity In Vitro and Demonstration of an Endogenous Inhibitor", Proc. Natl. Acad. Sci. USA, 80, pp. 5203-07 (1983); R. M. Kramer et al., "Solubilization and Properties of Ca^{2+}- Dependent Human Platelet Phospholipase A_2", Biochim. Biophys. Acta, 878, pp. 394-403 (1986); M. A. Clark et al., "Leukotriene D_4 Treatment of Bovine Aortic Endothelial Cells and Murine Smooth Muscle Cells in Culture Results in an Increase in Phospholipase A_2 Activity", J. Biol. Chem., 261, pp. 10713-18 (1986); L. A. Loeb and R. W. Gross, "Identification and Purification of Sheep Platelet Phospholipase A_2 Isoforms", J. Biol. Chem., 261, pp. 10467-70 (1986)).

Alternatively, phospholipase A_2 may be characterized by its reaction with a specific anti-

body in assays well-known in the art such as ELISA, Western Blots and immunoprecipitation.

Phospholipase A_2 purified according to this invention may be used to raise monoclonal or polyclonal antibodies. It may also be cleaved with various endo- and exopeptidases to produce the polypeptides of this invention. As demonstrated in the following examples, such purified protein was used as a source of amino acid sequence data to permit the synthesis of specific polypeptides which elicit site-specific anti-phospholipase A_2 antibodies. The amino acid sequence data was then employed to obtain nucleotide probes useful in isolating and selecting a DNA sequence encoding phospholipase A_2 from a genomic or cDNA library.

The amino acid sequence of phospholipase A_2 purified by the processes described above may be obtained by directly sequencing the material recovered from reverse phase HPLC according to this invention. More preferably, and according to an alternate embodiment of the present invention, the phospholipase A_2 is first subjected to discontinuous SDS-polyacrylamide gel electrophoresis (U. K. Laemmli, "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, Nature, 227, pp. 680-85 (1970)). This allows the protein to be separated from other potential protein contaminants. Following electrophoresis, the separated protein(s) are transferred to a solid support. The solid support should be compatible with a protein sequencer. Preferred solid supports are activated glass filters or, more preferably, polyvinylidene difluoride ("PVDF") membranes. The transfer of electrophoresed phospholipase A_2 to the support may be achieved by capillary transfer, or more preferably, by electrophoretic transfer. Following transfer, proteins may be visualized with any of a

number of well-known protein stains, such as Coomassie Blue R-250. The major phospholipase A_2 band is excised from the membrane and subjected to protein sequencing.

Protein sequencing may be achieved by standard techniques, preferably using automated Edman degradation, such as with an Applied Biosystems 470A gas phase sequencer. The amino terminal 19 amino acids of both human rheumatoid synovial fluid phospholipase A_2 and human platelet phospholipase A_2 purified according to this invention are both characterized by the amino acid sequence: H_2N-Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu. Such 100% homology suggests that other human inflammatory phospholipases A_2 may also contain this sequence. This amino terminal sequence differs from that of any phospholipase A_2 that has been purified previously from either human or non-human sources.

Due to limitations in the number of amino acids that can be accurately sequenced by standard techniques, the entire amino acid sequence of phospholipase A_2 is most preferably derived from the nucleotide sequence of a full-length human inflammatory phospholipase A_2 DNA or cDNA clone. The amino acid sequence obtained from the purified natural product has been used to confirm the identification of phospholipase A_2 clones isolated according to the processes of the present invention.

This invention also relates to polypeptides which correspond in amino acid sequence to at least a portion of the amino terminal 19 amino acids of human inflammatory phospholipase A_2. These polypeptides may be used to immunize animals and raise specific antibodies. Antibodies to small, weakly immunogenic polypeptides may be elicited by cross-linking the polypeptide to a carrier prior to injec-

tion into an animal. Many such carrier molecules are known in the art and include, but are not limited to, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and cytochrome c. Methods for cross-linking are also well known in the art and include the use of bifunctional cross-linking reagents, such as glutaraldehyde.

Such antibodies, as well as antibodies to intact natural phospholipase A_2 or to recombinant phospholipase A_2, are useful in humans and other mammals as anti-inflammatory therapeutics and disease modifying agents in diseases where pathogenic phospholipase A_2 has been implicated. The methods of treatment and their dosage levels and requirements are well recognized in the art and they may be chosen by those of skill in the art from available methods and techniques. For example, the antibodies may be combined with a pharmaceutically acceptable adjuvant for administration to a patient in an amount effective to provide anti-inflammatory effects and accordingly to lessen the severity and course of symptoms. The dosage and treatment regimens will depend upon factors such as the patient's health status, the severity and course of symptoms and the judgment of the treating physician.

Diseases which may be treated by compositions characterized by antibodies to phospholipase A_2 include, viral and bacterial infections, skin and connective tissue diseases, such as psoriasis, gastrointestinal disorders, such as pancreatitis and ulcers, ischemias, myocardial infarction, atherosclerosis, pulmonary dysfunctions, such as asthma, acute respiratory distress syndrome and alveolar proteinosis, septic shock, thrombosis, multiple sclerosis, demyelinating diseases and rheumatoid arthritis. These antibodies may also be employed as diagnostics in determining phospholipase A_2 levels

in tissues, body fluids, inflammatory cells and other cells using any conventional immunoassay technique. Phospholipase A_2 purified by the process of this invention, or recombinantly made, may be used in drug screening programs designed to search for inhibitors that can be used as anti-inflammatory, anti-arthritic and anti-thrombotic agents.

The present invention also relates to DNA sequences which encode all or a portion of the amino terminal 19 amino acids of human inflammatory phospholipase A_2. Such DNA sequences are preferably synthesized as a combination of oligonucleotides to account for the degeneracy of the genetic code. These DNA sequences, individually or in combination, are useful as probes to permit the isolation and selection of DNA sequences coding for intact phospholipase A_2 and phospholipase A_2-like polypeptides from various DNA and cDNA libraries, the synthesis of which is well-known in the art. Such DNA sequences, defined herein as "PLA_2 inserts", recombinant molecules including them and unicellular hosts transformed with them may be employed to produce large amounts of phospholipase A_2, substantially free from other proteins of human origin. Such libraries include chromosomal gene banks and cDNA or DNA libraries prepared from tissue or cell lines that are demonstrated to produce phospholipase A_2. These cell lines, as well as techniques for constructing DNA and cDNA libraries, are well known in the art.

The DNA sequences of the present invention either intact or portions thereof, are also useful to probe phospholipase A_2 mRNA levels in inflammatory cells (e.g., neutrophils, monocytes, lymphocytes) and many other cells (e.g., synoviocytes, endothelial cells, smooth muscle cells).

For the purpose of this application, phospholipase A_2-like polypeptides are defined as poly-

peptides which 1) are recognized by antibodies to native phospholipase A_2 in any standard immunoassay, or 2) will elicit antibodies which recognize native phospholipase A_2 in any standard immunoassay, or 3) demonstrate phospholipase A_2 enzymatic activity.

It should be understood that a variety of cloning and selection techniques might theoretically be useful in locating and identifying DNA or cDNA sequences of this invention that encode phospholipase A_2 other than the hybridization of oligonucleotides to genomic clones illustrated in the following examples. [See e.g., T. Maniatis et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor (1982).] If these alternate techniques do not yield a DNA or cDNA clone which encodes the entire phospholipase A_2 polypeptide, the selected DNA sequences may themselves be used as probes to select other DNA sequences coding full-length phospholipase A_2.

Partial or full-length DNA or cDNA sequences may be used in appropriate recombinant DNA molecules to transform appropriate eukaryotic and prokaryotic hosts for the production of the phospholipase A_2 and phospholipase A_2-like polypeptides encoded by them.

The DNA sequences and recombinant DNA molecules of the present invention may be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E.coli including col El, pCRl, pBR322, pMB9 and RP4, phage DNAs, e.g., the numerous derivatives of λ phage, e.g., NM 989, and other DNA phages, e.g., M13 and other filamentous single-stranded DNA phages, vectors useful in yeasts, such as the 2μ plasmid, vectors useful in animal cells, such as those containing SV-40, adenovirus and retrovirus derived DNA

sequences (e.g., BG368 and BG341) and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other derivatives thereof.

Such expression vectors are also characterized by at least one expression control sequence that may be operatively linked to the phospholipase A_2 DNA sequence inserted in the vector in order to control and to regulate the expression of that cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage λ, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, the major late promoter of adenovirus, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Among such useful expression vectors are vectors that enable the expression of the cloned phospholipase A_2-related DNA sequences in eukaryotic hosts, such as animal and human cells [e.g., P. J. Southern and P. Berg, J. Mol. Appl. Genet., 1, pp. 327-41 (1982); S. Subramani et al., Mol. Cell. Biol., 1, pp. 854-64 (1981); R. J. Kaufmann and P. A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol Biol., 159, pp. 601-21 (1982); R. J. Kaufmann and P. A. Sharp, Mol. Cell. Biol., 159, pp. 601-64 (1982) S. I. Scahill et al., "Expression And Characterization Of The Product Of A Human Immune Interferon

DNA Gene In Chinese Hamster Ovary Cells", Proc. Natl. Acad. Sci. U.S.A., 80, pp. 4654-59 (1983); G. Urlaub and L. A. Chasin, Proc. Natl. Acad. Sci. USA, 77, pp. 4216-20 (1980)].

Furthermore, within each specific expression vector, various sites may be selected for insertion of the PLA_2 inserts of this invention. These sites are usually designated by the restriction endonuclease which cuts them. They are well recognized by those of skill in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vector could be joined to the fragment by alternative means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment and its operative linking therein to an expression control sequence, is determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination or binding of the protein to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a DNA sequence is determined by a balance of these factors, not all selections being equally effective for a given case.

Useful expression hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, such as E.coli SG-936, E.coli HB 101, E.coli W3110, E.coli X1776, E.coli X2282, E.coli MC1061, E.coli DHI, and E.coli MRC1, Pseudomonas, Bacillus, such as Bacillus subtilis,

Streptomyces, yeasts and other fungi, animal cells, such as COS cells and CHO cells, human cells, insect cells and plant cells in tissue culture.

Of course, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention or in producing the phospholipase A_2-like polypeptides. However, a particular selection of a host-expression vector combination may be made by those of skill in the art after due consideration of the principles set forth herein without departing from the scope of this invention. For example, the selection should be based on a balancing of a number of factors. These include, for example, compatibility of the host and vector, toxicity of the proteins encoded by the DNA sequence to the host, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, biosafety, costs and the folding, form or any other necessary post-expression modifications of the desired protein.

Alternatively, if the isolated genomic clone contains phospholipase A_2 expression control sequences, such as promoters, ribosome binding sites, and polyadenylation signals in addition to the phospholipase A_2 coding sequence, expression vectors may be unnecessary. In this case, the genomic clone alone may be used to transfect eukaryotic hosts, which, in turn can express phospholipase A_2. Such hosts include cells well-known in the art, such as mouse L-cells or CHO cells.

Thus, the present invention provides three different mehtods in which to produce recombinant phospholipase A_2-like polypeptides. These methods include heterologous promoter-mediated expression of phospholipase A_2 cDNAs, heterologous promoter-regulated expression of phospholipase A_2 genomic DNA, and native

promoter-mediated expression of phospholipase A_2 genomic DNA. The latter two methods are only applicable to eukaryotic cells that are able to perform proper splicing out of introns.

It should be understood that in addition to the DNA and cDNA sequences described herein, the present invention also relates to DNA sequences which hybridize to the foregoing DNA sequences, as well as DNA sequences which, due to the degeneracy of the genetic code, code on expression for human phospholipase A_2-like polypeptides coded for on expression by the foregoing DNA or cDNA sequences.

In order that our invention herein described may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and should not be construed as limiting this invention in any way to the specific embodiments recited therein.

EXAMPLE I

Purification Of Phospholipase A_2 From Human Platelets

According to one embodiment of this invention, we purified phospholipase A_2 from human platelets as follows:

A. Preparation Of Human Platelets

We centrifuged a preparation of normal human platelet concentrates (obtained from the Red Cross; within 5 days of phlebotomy) at 200xg in a Sorvall RT6000B centrifuge for 10 min at room temperature to remove any residual red cells. The supernatant was made 1 mM in EGTA and the platelets pelleted by centrifugation at 2,500xg for 15 min at 4°C. We washed the pellets with 120 mM NaCl, 2 mM EGTA, 30 mM Tris-HCl, pH 7.4 and then resuspended the platelets in that buffer at a protein concentra-

tion of 10 mg/ml. These preparations were quick frozen in a dry ice-acetone bath and stored in 10 ml aliquots at -70°C before further use.

B. Acid-Extraction Of Human Platelets

We thawed 750 ml of the above-prepared platelets (7510 mg protein) and sonicated them in three batches at 4°C with a probe sonicator using a 3-4 inch standard horn (Model W-225; Heat Systems-Ultrasonics). Each batch received six 15 second pulses at an output setting of 6 with a 45 second interval between each pulse. The sonicates were pooled and mixed with an equal volume of ice-cold 0.36 N sulfuric acid and let stand at 4°C for 60 min. Precipitated material was separated by centrifugation at 10,000xg for 30 min at 4°C. We collected the supernatant and reextracted the pellets in a total of 500 ml of 0.18 N sulfuric acid containing 150 mM NaCl on ice for 60 min. The remaining insoluble material was pelleted by centrifugation as above. The supernatants from both extracts were pooled (2000 ml) and dialyzed overnight against 3 x 16 liters of 200 mM NaCl, 50 mM sodium acetate, pH 4.5 using Spectra/Por membranes (3500 dalton molecular weight cutoff). We centrifuged the dialyzed platelet preparation at 15,000xg for 40 min at 4°C to remove any precipitated material.

C. Partial Purification Of Human Platelet PLA_2 Activity By Cation Exchange Chromatography

We applied the supernatant (718 mg protein) to a 1.6 x 27 cm Fast S Sepharose (Pharmacia) column that had been pre-equilibrated with 200 mM NaCl, 50 mM sodium acetate, pH 4.5 at a flow rate of 90 ml/h. After washing the column with 150 ml of the same buffer, we developed it with 550 ml of a linear salt gradient from 200 mM - 2 M NaCl in 50 mM sodium ace-

tate, pH 4.5. Fractions (5 ml) were collected and assayed for absorbance at 280 nm and for phospholipase A_2 activity. Figure 1 shows that the phospholipase A_2 eluted with approximately 1 M NaCl at fractions 74-82. We pooled these fractions and concentrated them to 0.8 ml using an Amicon ultrafiltration stirred cell with a YM 5 membrane.

D. Partial Purification Of Human Platelet PLA_2 Activity By Gel Filtration

We then chromatographed the concentrated peak fractions from the cation exchange column on a Sephadex G-50 superfine (Pharmacia) column (1 x 48 cm) which had been pre-equilibrated in 500 mM NaCl, 50 mM sodium acetate, pH 4.5. We collected 0.5 ml fractions at a flow rate of 2 ml/h. Fractions were assayed for absorbance at 280 nm and for phospholipase A_2 activity. Figure 2 demonstrates that enzymatic activity eluted in fractions 45-56, with an apparent molecular weight of 13,000 daltons.

E. Purification Of Human Platelet PLA_2 By Reverse-Phase HPLC

The pooled peak fractions from the gel filtration column (6 ml; 100 µg protein) were further purified on a C4 reverse-phase HPLC column (Vydac; 0.46 x 25 cm) that was equilibrated at 29°C with 0.1% trifluoroacetic acid (TFA). The reverse-phase column was developed at a flow rate of 1 ml/min with a 45 minute gradient (0-75% acetonitrile in 0.1% TFA), collecting 0.5 ml fractions. The column eluate was monitored for absorbance at 214 nm (AFU 0.2) and 280 nm (AFU 0.05). An aliquot of each fraction was diluted into 500 mM NaCl, 50 mM acetate, pH 4.5 containing 1 mg/ml bovine serum albumin (Sigma) and assayed for activity. Figure 3, panel B, demonstrates that approximately 35% of the applied phospholipase

A_2 activity was recovered in a single peak contained in fraction 45. Figure 3, panel A, indicates the activity eluted at about 32% acetonitrile. We added 1 µl of 5% SDS to this fraction and dried the sample in a Speed-Vac concentrator (Savant).

Figure 4 depicts, in tabular form, the entire purification process. The final yield of phospholipase A_2 from platelets was 34% and the protein was purified over 1,100,000-fold over the starting material. After extraction and dialysis, the total phospholipase A_2 activity increased 63-fold over that observed in the sonicate and was assumed to be 100%. The purification-fold was estimated assuming 100% recovery of enzymatic activity during these steps.

F. SDS-PAGE/Electroblotting Onto PVDF Membrane Of Human Platelet PLA_2

We dissolved the PLA_2 protein in 25 µl of electrophoresis sample buffer, incubated the sample for 10 min at 60°C, loaded it onto a minigel (5 x 7 cm) containing a 16% SDS-polyacrylamide gel and a 5% stacking gel and electrophoresed at 20 mA constant current for 120 min (U. K. Laemmli, supra). Following electrophoresis, we soaked the gel in transfer buffer (10 mM 3-[cyclohexylamino]- 1-propanesulfonic acid, 10% methanol, 0.05% SDS, pH 11.25) for 5 min and electroblotted onto a PVDF membrane (Immobilon; 0.45 µm pore size, Millipore) for 1 h at 150 mA (P. Matsudaira, "Sequence from Picomole Quantities of Proteins Electroblotted Onto Polyvinylidene Difluoride Membranes", J. Biol. Chem., 262, pp. 10035-38 (1987)). We visualized the proteins by staining the membrane with Coomassie Blue R-250 (Figure 5). We then rinsed the membrane extensively with deionized water, dried it and stored it at -20°C.

EXAMPLE 2

Purification Of Phospholipase A_2 From Human Synovial Fluid

According to one embodiment of this invention, we purified phospholipase A_2 from human synovial fluid as follows:

A. Preparation Of Human Rheumatoid Synovial Fluid

Synovial fluid was aspirated from patients diagnosed with classical rheumatoid arthritis, as defined by American Rheumatism Association criteria. We removed cells and debris from synovial fluids by centrifugation at 4°C for 20 min at 3,000xg in a Sorvall RC3B centrifuge. The synovial fluids were stored at -70°C before further use.

B. Acid Extraction Of Rheumatoid Synovial Fluid

We thawed the synovial fluids, pooled them to yield 50 ml (1932 mg protein) and mixed them with an equal volume of 0.36 N sulfuric acid. To this we added 100 ml of 0.18 N sulfuric acid containing 150 mM NaCl and incubated the mixture on ice for 60 min. The mixture was then dialyzed overnight against 2 x 4 liters of 200 mM NaCl, 50 mM Na acetate, pH 4.5 (Spectra-Por membranes; 3500 dalton cutoff). We then removed precipitated material by centrifuging at 15,000xg for 40 min at 4°C.

C. Partial Purification Of Human Synovial Fluid PLA_2 Activity By Cation Exchange Chromatography

We applied the supernatant (1582 mg protein) to a 1.6 x 27 cm Fast S Sepharose (Pharmacia) column that had been pre-equilibrated with 200 mM NaCl, 50 mM sodium acetate, pH 4.5 at a flow rate of 90 ml/h. After we washed the column with 150 ml of

the same buffer, we developed with 550 ml of a linear salt gradient from 200 mM - 2 M NaCl in 50 mM Na acetate pH 4.5. Fractions (5 ml) were collected and assayed for protein content, by measuring absorbance at 280 nm and for phospholipase A_2 activity. Figure 6 shows that the protein eluted from the column with approximately 1 M NaCl at fractions 84-98. We pooled these fractions and concentrated them to 0.8 ml using an Amicon ultrafiltration stirred cell with a YM 5 membrane.

D. Purification Of Human Rheumatoid Synovial Fluid PLA_2 Activity By Gel Filtration

We then chromatographed the concentrated peak fractions from the cation exchange column (2.5 mg protein) on a Sephadex G-50 superfine (Pharmacia) column (1 x 48 cm) which had been pre-equilibrated in 500 mM NaCl, 50 mM Na acetate, pH 4.5. We collected 0.5 ml fractions at a flow rate of 2 ml/h. Fractions were assayed for absorbance at 280 nm and for phospholipase A_2 activity. Figure 7 demonstrates that enzymatic activity eluted in fractions 45-56, with an apparent molecular weight of 13,000 daltons.

E. Purification Of Human Rheumatoid Synovial Fluid PLA_2 By Reverse Phase HPLC

The pooled peak fractions from the gel filtration column (6 ml; 100 µg protein) were further purified on a C4 reverse-phase HPLC column (Vydac; 0.46 x 25 cm) that was equilibrated at 29°C with 0.1% trifluoroacetic acid (TFA). The column was then developed at a flow rate of 1 ml/min with a 45 minute gradient (0-75% acetonitrile in 0.1% TFA), collecting 0.5 ml fractions. The column eluate was monitored at 214 nm (AUF 0.2) and 280 nm (AUF 0.05). An aliquot of each fraction was diluted into 500 mM NaCl, 50 mM acetate, pH 4.5 buffer containing 1 mg/ml

bovine serum albumin and assayed for phospholipase A_2 activity. Figure 8, panel B, demonstrates that the phospholipase A_2 activity was recovered in a single peak contained in fractions 48 and 49. Figure 8, panel A, indicates the activity eluted at about 30% acetonitrile. We added 1 µl of 5% SDS to these fractions and dried the samples in a Speed-Vac concentrator (Savant).

Figure 9 demonstrates, in tabular form, the entire purification process. The final yield of phospholipase A_2 from rheumatoid synovial fluid was 57% and the protein was purified over 100,000-fold over the starting material.

F. SDS PAGE/Electroblotting Onto PVDF Membrane Of Human Rheumatoid Synovial Fluid PLA_2

We dissolved the PLA_2 protein in 25 µl of electrophoresis sample buffer, incubated the sample for 10 min at 60°C, loaded it onto a minigel (5 x 7cm) containing a 16% SDS-polyacrylamide gel and a 5% stacking gel and electrophoresed at 20 mA constant current for 120 min. Following electrophoresis, we soaked the gel in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, 0.05% SDS, pH 11.25) for 5 min and electroblotted onto a PVDF membrane (Immobilon; 0.45 um pore size, Millipore) for 1 h at 150 mA. We visualized the proteins by staining the membrane with Coomassie Blue R-250 (Figure 10). We then rinsed the membrane extensively with deionized water, dried it and stored it at -20°C.

EXAMPLE 3

Preparation Of Substrate And Assay of Phospholipase A_2 Activity

We prepared the substrate, ^{3}H-oleic acid-labelled E.coli, as follows: We grew an overnight

culture of E.coli in 1% bactotryptone, 0.5% NaCl, diluted it 1:20 into fresh broth and allowed the cells to regrow, monitoring cell growth with a Klett-Summerson colorimeter, until the absorbance reached 40. We then added 1/100th of a volume of 10% Brij 35 (Sigma Chemicals) and 1/200th of a volume of ^{3}H-oleic acid (9,10-^{3}H-[N]-oleic acid, New England Nuclear) at 10 mCi/ml to the culture. After 5h of growth, we autoclaved the culture and stored the bacteria overnight at 4°C. We then pelleted the bacteria by centrifugation (16,000 rpm, 30 min, 4°C, SS34 rotor), combined the loose pellets and washed 4 times in 0.7 M Tris-HCl, 10 mM $CaCl_2$, 0.1% BSA, pH 8.0, until radioactivity in the supernatant was low. The bacteria were stored in this buffer containing 0.2% Na azide at 4°C. We then prepared, for example, a 400 ml culture labeled with 20 mCi of ^{3}H-oleic acid. This yielded about 7 x 10^{8} counts per minute or about 10% of the input counts in labeled bacteria. Prior to use in an assay, we washed aliquots of cells for 30 min on ice in 200 mM Tris-HCl, 12 mM EDTA, pH 8.0, followed by 25 mM Tris-HCl, pH 8.0. A typical assay used, for each point, 100,000 cpm, which was added in a volume of 25 µl.

We performed a typical phospholipase A_2 assay as follows: Samples to be assayed for phospholipase A_2 activity (20 µl) were mixed with 25 µl of autoclaved [^{3}H]-oleic acid-labeled E.coli as substrate and brought to a total volume of 200 µl with 0.1 M Tris-HCl, pH 9, containing 10 mM $CaCl_2$, 1 mM 2-mercaptoethanol and 1 mg/ml BSA. The reaction was incubated at 37°C for 15 min and stopped by the addition of 100 µl of 2N HCl, followed by 100 µl of delipidated BSA. Samples were vortexed and incubated on ice for 30 min. The samples were spun in an Eppendorf microcentrifuge for 5 min at 10,000xg and 250 µl of the supernatants containing released

[^{3}H]oleic acid were counted for radioactivity after mixing with 4 ml of scintillation fluid compatible with aqueous solutions (Fisher). A unit of activity for human rheumatoid synovial fluid phospholipase A_2 was defined as the amount of protein necessary to release 1 x 10^9 cpm of [^{3}H]-oleate in 15 min at 37°C. A unit of activity for human platelet phospholipase A_2 was defined as the amount of protein necessary to release 1 x 10^6 cpm of [^{3}H]-oleate in 15 min at 37°C.

EXAMPLE 4

Amino Acid Sequence Analysis

The major band visualized on the PVDF membrane following electrophoresis and electroblotting was excised and subjected to automated Edman degradation (P. Matsudaira, J. Biol. Chem., 262, pp. 10035-38 (1987)) using an Applied Biosystems 470A gas phase protein sequencer equipped with a model 900A data system (R. M. Hewick et al., "A Gas-Liquid Solid Phase Peptide and Protein Sequenator", J. Biol. Chem., 256, pp. 7990-97 (1981)). The resulting phenylthiohydantoin amino acids were analyzed on-line using an Applied Biosystems 120A PTH amino acid analyzer equipped with a PTH-C18 column (2.1 x 220 mm). The amino terminal 19 amino acids were determined for both platelet and synovial fluid phospholipase A_2. Both proteins had the identical amino terminal sequence of H_2N-Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu.

EXAMPLE 5

Synthesis Of Human Inflammatory Phospholipase A_2 Polypeptides

We synthesized a 16 amino acid polypeptide, Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-

Gly-Lys-Glu-Ala, corresponding to amino acids 2-17 of the above-sequenced phospholipases A_2. We prepared the polypeptide by solid-phase synthesis employing an Applied Biosystems 430A Peptide Synthesizer, using the procedures of R. B. Merrifield, "Solid Phase Peptide Synthesis. I. Synthesis of a Tetrapeptide", J. Amer. Chem. Soc., 85, pp. 2149-54 (1963)

The polypeptide was coupled to keyhole limpet hemocyanin with glutaraldehyde before being used to immunize rabbits. For primary injection, 1.5 mg of the polypeptide-KLH complex was emulsified with Freund's complete adjuvant and administered intramuscularly. For subsequent injections, 0.75 mg of the polypeptide-KLH complex was emulsified with Freund's incomplete adjuvant and administered intramuscularly. Animals were bled every 2 weeks and sera assayed for anti-human inflammatory phospholipase A_2 titer by ELISA.

Other phospholipase A_2-like polypeptides, as well as mature phospholipase A_2 may be similarly synthesized and used to raise anti-human inflammatory phospholipase A_2 antibodies. Additionally, phospholipase A_2-like polypeptides of sufficient size and immunogenicity may be used directly to elicit antibodies to phospholipase A_2 without coupling to KLH.

EXAMPLE 6

Synthesis Of Human Inflammatory Phospholipase A_2 Oligonucleotides

Unless otherwise specified, all molecular biological techniques are described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor, New York (1982).

We synthesized a number of oligonucleotides based on three different hexapeptides of the deduced

amino acid sequence of human inflammatory phospholipase A_2. The three hexapeptides were: Asn-Phe-His-Arg-Met-Ile, Met-Ile-Lys-Leu-Thr-Thr, and Thr-Thr-Gly-Lys-Glu-Ala. More than one nucleotide was introduced at various positions during the synthesis of these oligonucleotides, to account for the degeneracy of the genetic code. Thus, the product of any single oligonucleotide synthesis was actually a mixture of oligonucleotides, all of which potentially coded for the corresponding hexapeptide. For the purposes of the present specification and claims, the following code is used to designate nucleotides:

A- adenine
N- adenine, thymidine, guanidine, or cytidine
T- thymidine
Pu- adenine or guanidine
G- guanidine
Py- thymidine or cytidine
C- cytidine
Z- adenine, guanidine or thymidine

The following oligonucleotides based on the hexapeptide Asn-Phe-His-Arg-Met-Ile were synthesized:

PLA_2-06: 5' ATC ATPu CGPu TGPu AAPu TT 3'
PLA_2-07: 5' ATC ATPy CGPu TGPu AAPu TT 3'
PLA_2-08: 5' ATC ATPy CTPu TGPu AAPu TT 3'

The following oligonucleotides based on the hexapeptide Met-Ile-Lys-Leu-Thr-Thr were synthesized:

PLA_2-09: 5' GTN GTPy AAPy TTZ ATC AT 3'
PLA_2-10: 5' GTN GTPu AGPy TTZ ATC AT 3'
PLA_2-11: 5' GTN GTPy AGPy TTZ ATC AT 3'

The following oligonucleotides based on the hexapeptide Thr-Thr-Gly-Lys-Glu-Ala were synthesized:

PLA_2-12: 5' GCPy TCPy TTPu CCPu GTPu GT 3'
PLA_2-13: 5' GCPy TCPy TTPy CCPy GTPy GT 3'

All of the oligonucleotides were synthesized on an Applied Biosystems 380A automated DNA

synthesizer, using the procedure described by L. J. McBride and M. H. Caruthers, "The Synthesis of Oligodeoxypyrimidines on a Polymer Support", Tetrahedron Letters, 24, pp. 245-48 (1983).

EXAMPLE 7

Construction Of A Human Genomic Library

High molecular weight DNA was isolated from monolayer cultures of a mutant fibroblast cell line which contains 5 copies of the X chromosome (Human Genetic Mutant Cell Repository, Camden, New Jersey; repository number GM5009) using standard techniques. We then partially digested the DNA with restriction enzyme Sau3A and dephosphorylated the fragments with bacterial alkaline phosphatase. Subsequently, we ligated the fragments to BamHI-digested λEMBL3 DNA (A.-M. Frischauf et al, "Lambda Replacement Vectors Carrying Polylinker Sequences", J. Mol. Biol., 170, 827-42 (1983)) and packaged the bacteriophage genomes using a two-extract kit according to manufacturer's directions (Amersham Corp.). Recombinant bacteriophage were selected by plating on E.coli MP801 cells (a gift of Dr. Mark Pasek, Biogen Inc., Cambridge, MA), a P2 lysogen of SG4119.

EXAMPLE 8

Screening A Human Genomic Library For Inflammatory Phospholipase A_2 Sequences

We initially screened the GM5009 human genomic DNA EMBL3 phage library for phospholipase A_2 sequences with a combination of oligonucleotides PLA_2-06, PLA_2-07 and PLA_2-08 by plaque hybridization screening (S.L.C. Woo, "A Sensitive and Rapid Technique for Recombinant Phage Screening", Meth. Enzymol., 68, pp. 389-96 (1979)).

We grew a culture of E.coli LE392 cells in L-broth plus 0.2% maltose overnight at 37°C. We then pelleted the cells by centrifugation and resuspended the cell pellet in an equal volume of SM buffer. For each plate, we pre-adsorbed 0.9 ml of cells with 2 x 10^5 phage particles at room temperature for 15 min. We then added 50 ml of L-broth plus 10 mM $MgSO_4$ and 0.7% agarose (melted and held at 55°C) and plated the mixture onto ten LB-$MgSO_4$ Nunc plates (25 cm x 25 cm). The plates were incubated at 37°C for 8 h or until the plaques were just nearly touching. The plates were then chilled at 4°C to allow the agarose to harden.

We presoaked Genescreen Plus filters (New England Nuclear) in a 1:10 dilution of an overnight culture of LE392 cells for 10 min at room temperature in order to coat each filter with bacteria. After air drying, the filters were contacted with the plates containing the recombinant plaques for 5 min. The filters were removed and placed phage-side up onto LB plus 10 mM $MgSO_4$ plates. A second replica lift was made from each plate by the same procedure. We then incubated all filters at 37°C for 5 h. After incubation, we removed the filters from the plates and placed them in a pool of 0.5 N NaOH, 1.5 M NaCl, two times, to lyse the phage. The filters were then neutralized in 0.5 M Tris-HCl pH 7.0, 1.5 M NaCl and scrubbed free of cell debris.

We ^{32}P-labeled a combination of oligonucleotides PLA_2-06, PLA_2-07 and PLA_2-08 with polynucleotide kinase and high specific activity ^{32}P-ATP using standard techniques. We pre-hybridized the filters in plaque screen buffer for 1 h and then hybridized to the above labeled probes at 45°C for 15 h in plaque screen buffer containing 10% dextran sulfate and 100 µg/ml yeast tRNA according to the

manufacturer's specifications for plaque screen membranes (New England Nuclear).

The filters were then washed two times in plaque screen buffer at 45°C, followed by two washes in 3.2 M tetramethylammonium chloride "(TMACl)", 1% SDS at 45°C (P. E. Devlin et al, "Southern Analysis of Genomic DNA With Unique and Degenerate Oligonucleotide Probes: A Method for Reducing Probe Degeneracy", DNA, (in press) (1988)). Positive phage were detected by autoradiography. We selected 64 positive plaques by this technique. Agarose plugs containing the positive plaques were removed from the master plate, transferred into SM buffer and 18 of these were rescreened at lower density using the same technique. For rescreening, we used nitrocellulose filters and included a final wash in 1 M ammonium acetate following neutralization in Tris-HCl-NaCl. The filters were baked at 80°C for 2 h following this wash.

Ten of the rescreened clones remained positive and at least one clone also hybridized to a combination of probes PLA_2-09, PLA_2-10 and PLA_2-11 which had been ^{32}P-labeled with polynucleotide kinase. This clone is referred to as PLA_2 8.5 EMBL3.

We isolated DNA from clone PLA_2 8.5 EMBL3 and determined the insert to be 16 kilobases (kb) upon restriction enzyme digestion analysis. Using the Southern Blot technique (E. M. Southern et al., "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis", J. Mol. Biol., 98, pp. 503-18 (1975)) we determined that the sets of probes PLA_2-06 to PLA_2-08 and PLA_2-09 to PLA_2-11 all hybridized to a single 6.2 kb HindIII fragment of PLA_2 8.5 EMBL3 ("the 6.2 kb PLA_2 insert"). This fragment was isolated following digestion of the clone with HindIII and SalI and subsequent electrophoresis in a 0.8% low-melting agarose gel. The

actual 6.2 kb HindIII fragment isolated did not contain a SalI site, but this digestion was necessary to eliminate a similarly-sized HindIII fragment of PLA_2 8.5 EMBL3. This latter fragment contains a SalI site and thus the double digestion eliminates the possibility of contamination.

The fragment was cloned into HindIII-digested pNN01 that had been treated with calf intestinal alkaline phosphatase. We constructed the sequencing plasmid pNN01 by removing the synthetic polylinker from the commercially available plasmid pUC8 (Pharmacia PL Biochemicals) by restriction digestion and replacing it with a new synthetic segment. The 2.5 kb backbone common to the pUC plasmids, which provides an origin of replication and confers ampicillin resistance, remained unchanged. The novel synthetic portion of pNN01 is shown in Figure 11A.

For ligation, we mixed 10 ng of digested vector with 40 ng of the 6.2 kb PLA_2 insert in 100 µl of T4 DNA ligase buffer containing 400 units of T4 ligase. Ligation was achieved by incubation at room temperature for 5 h. We used 20 µl of the ligation mixture to transform 0.2 ml of competent E.coli MC1061 cells. Transformants were grown on LB + ampicillin plates at 37°C overnight. We isolated plasmid DNA from 12 potential transformants using the alkaline miniprep procedure (T. Maniatis et al., supra) and determined that one transformant, PSQ 130, contained the 6.2 kb PLA_2 insert (Figure 11B). The insert was then sequenced using the method of Maxam and Gilbert (A. M. Maxam and W. Gilbert, "A New Method for Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-64 (1977)). A BamHI-NcoI fragment of the 6.2 kb PLA_2 insert, which hybridized to the PLA_2 oligonucleotide probes of this invention, had the following nucleotide sequence:

C CAT GGG AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC.

This sequence encodes the polypeptide:

His Gly Asn Leu Val Asn Phe His Arg Met Ile Lys Leu Thr Thr Gly Lys Glu Ala Ala Leu Ser Tyr Gly Phe Tyr Gly Cys His Cys Gly Val Gly Gly Arg Gly Ser.

The underscored 19 amino acids of this polypeptide correspond exactly with the protein sequence information obtained from purified human platelet and rheumatoid synovial fluid phospholipase A_2, confirming that we had isolated at least part of the genomic clone. The additional 3' end region of this region encodes amino acids that correspond to conserved sequences in other phospholipases A_2 that have been sequenced.

The entire DNA sequence of the 6.2 kb PLA_2 insert as well as the amino acid sequence coded for in the exons is shown in Figure 12. Potential intron splice sites are indicated by arrowheads. The coding sequence for mature phospholipase A_2 begins at nucleotide 2722 (arrow, Figure 12) and is contained within exon 2. Exon 2 begins at nucleotide 2702 and encodes 6 in-frame amino acids preceding the amino terminal asparagine residue of mature phospholipase A_2. We believe that these 6 amino acids encode the carboxy terminal 6 amino acids of the phospholipase A_2 signal sequence.

An open reading frame of 14 amino acids beginning with a methionine residue and having characteristic properties of a signal sequence is located between nucleotides 2453 and 2492. This nucleotide sequence also terminates with a characteristic GT splice site at nucleotides 2493-2494. It is unlikely that this represents the N-terminal portion of the

in vivo signal, because no promoter-like sequences are found within the 100 nucleotides located 5' to this region.

Exons 3 and 4, which encode the remainder of phospholipase A_2 and an in-frame stop codon, are located at nucleotides 3105-3211 and 5383-5523, respectively. The putative polyadenylation signal, AATAAA, is located at nucleotides 5771-5776 (underscored in Figure 12).

The mature PLA_2 polypeptide coded for by exons 2, 3 and 4 consists of 124 amino acids and has the formula: NLVNFHRMIK LTTGKEAALS YGFYGCHCGV GGRGSPKDAT DRCCVTHDCC YKRLEKRGCG TKFLSYKFSN SGSRITCAKQ DSCRSQLCEC DKAAATCFAR NKTTYNKKYQ YYSNKHCRGS TPRC.

In the above-cited formula as well as throughout this application the amino acids are represented by single letter codes as follows:

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

We believe that the 3 exons of the genomic clone contained within the 6.2 kb PLA_2 EMBL3 8.5 HindIII fragment encode phospholipase A_2) based on the following observations. First, the clone encodes the identical N-terminal amino acid sequence identified for the purified native enzyme by protein sequencing (see Example 4). This sequence represents an amphiphilic alpha-helix that is typical for all phospholipases A_2 sequenced to date. It also encodes the highly conserved lipophilic residues within this alpha-helix (e.g., Leu_2, Phe_5 and Ile_9). Furthermore, the clone codes for a cluster of basic amino acids (e.g., Arg_7, Lys_{11} and Lys_{15}) which is

believed to be an important determinant in the interaction of phospholipase A_2 with specific biological targets. Second, the clone encodes a characteristic stretch of amino acids Tyr_{25}-Gly-Cys-X-Cys-Gly-X-Gly-Gly-X-X-X-Pro_{37} and Asp_{49}, where X is any amino acid, that are part of the calcium binding loop of phospholipases A_2. Finally, the clone encodes the characteristic amino acid residues that constitute the active site of all phospholipases A_2, namely His_{48}, Asp_{99}, Tyr_{52} and Tyr_{73}.

The phospholipase A_2 amino acid sequence coded for by the 6.2 kb HindIII fragment of genomic DNA clone PLA_2 EMBL3 8.5 also exhibits the placement of half-cysteine residues that is typical for group II phospholipases A_2. Thus, it contains a cysteine residue at amino acid 50 and has an extension of several amino acid residues at the C-terminus which ends in a half cysteine. A comparison of the amino acid sequence of bovine pancreatic PLA_2, C. atrox venom PLA_2, and the sequence encoded by the 6.2 kb PLA_2 insert as well as a consensus sequence, is depicted in Figure 13.

EXAMPLE 9

Construction Of Vectors For The Expression Of Human Inflammatory Phospholipase A_2 In Animal Cells

In order to confirm that the 6.2 kb PLA_2 insert encodes a functional polypeptide and to enable us to obtain mRNA as a source of cDNA, we made several constructs for expression of this phospholipase A_2 sequence in animal cells.

Plasmid PLA_2 6.2 BG368 3(+) (Figure 14B) was synthesized as follows: PSQ 130 was digested with HindIII and the 6.2 kb PLA_2 insert was isolated by preparative gel electrophoresis through low-melt agarose. The gel slice containing this fragment was excised and stored at 4°C.

The animal cell expression parent vector, BG368, was constructed as follows: As depicted in Figure 14A, we cut animal cell expression vector BG312 [R. Cate et al., "Isolation Of The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986)] with EcoRI and BglII to delete one of each of the two EcoRI and the two BglII restriction sites (the EcoRI site at position 0 and the BglII site located at approximately position 99). The resulting plasmid, BG368, retained an EcoRI site in the cloning region and a BglII site after the cloning region. This left a single EcoRI site and a single BglII site in the polylinker for cloning purposes.

More specifically, we deleted one EcoRI site and one BglII site by sequential partial digestion of BG312 with restriction enzymes EcoRI and BglII, respectively. We filled in with Klenow fragment of E.coli polymerase and 4 nucleotides then religated to produce BG368, which contains unique restriction sites for EcoRI and BglII enzymes.

BG368 was linearized by digestion at the unique HindIII site in the polylinker region. BG368 contains the SV40 origin of replication and enhancer sequence, the adenovirus major late promoter, a polylinker region containing unique restriction sites for the insertion of DNA sequences for expression, the SV40 3' untranslated region, including the polyadenylation signal and the 3' splice site. We then treated the linearized vector with calf intestinal alkaline phosphatase to prevent reannealing, phenol extracted it and purified it by preparative gel electrophoresis as above.

Slices of low melt agarose containing either the vector or the insert were diluted with H_2O and melted at 65°C for 2-3 minutes. We ligated aliquots,

equivalent to 60 ng and 15 ng, respectively, of 6.2 kb PLA_2 insert and HindIII linearized BG368 vector with 400 units of T4 ligase in a total volume of 40 µl of 1X T4 ligase buffer overnight at room temperature. We used 20 µl of this ligation mixture to transform E.coli MC1061 cells. The transformants were grown on LB + amplicillin plates at 37°C overnight. We isolated plasmid DNA from 10 transformants using the alkaline miniprep procedure. By digesting the plasmids with restriction enzymes XhoI and NdeI we determined that three transformants contained the PLA_2 insert. This restriction enzyme analysis also allowed us to determine orientation of this insert with respect to the adenovirus major late promoter in this vector. We prepared large scale plasmid preparations of DNA from one transformant containing the + orientation, named PLA_2 6.2 BG368 3(+) and one containing the - orientation, named PLA_2 6.2 BG368 8(-).

We then prepared plasmid PLA_2 3.8 BG341(+) (Figure 14, panel C) as follows: PSQ 130 was digested with NotI to release the entire 6.2 kb PLA_2 insert in addition to the polylinker from pNN01 (Figure 11, panel B). We purified this NotI fragment by gel electrophoresis through 1% agarose in TBE buffer. The fragment was then electroeluted from the gel and recovered by ethanol precipitation. We then digested the NotI fragment with EagI yielding two fragments approximately 2.4 kb and 3.8 kb in length. We purified the 3.8 kb fragment ("the 3.8 kb PLA_2 insert") by low melt agarose gel electrophoresis as described above. From the nucleotide sequence of the PLA_2 insert we determined that the 3.8 kb PLA_2 insert contains a potential open reading frame encoding an initiating methionine as well as amino acids which are characteristic of signal sequences. This fragment also contains Exons 2, 3, and 4, as well as a

donor sequence for splicing the amino terminus of the signal sequence to Exon 2.

Plasmid BG341 (Figure 14D) was also derived from BG312 (R. Cate et al., Cell, 45, pp. 685-98 (1986)). After partial digestion of BG312 with BglII to linearize it at the BglII site located at approximately position 99, we filled in the BglII site with Klenow fragment of E.coli DNA polymerase and deoxyribonucleotides then religated the vector. We linearized the vector by partial digestion with BamHI at the site following the SV40 poly A region, filled in the site with Klenow and deoxyribonucleotides and religated the vector. The resultant vector with unique BglII and BamHI sites was linearized with SmaI and a linker having the sequence 5'GCGGCCGCGCTCGAGCTCG3' was ligated into the SmaI site, thus destroying it and creating a NotI site in the cloning region.

BG341 was then linearized by digestion at its unique NotI site. The linearized plasmid was then alkaline phosphatase treated and purified in an identical manner to that described above for BG368.

Slices of low melt agarose containing either the vector or the insert were diluted with H_2O and melted at 65°C for 2-3 minutes. We ligated aliquots equivalent to 60 ng and 15 ng respectively of 3.8 kb PLA_2 insert and NotI linearized BG341 vector with 400 units of T4 ligase in a total volume of 40 µl of 1X T4 ligase buffer overnight at room temperature. We used 20 µl of this ligation mixture to transform E.coli MC1061 cells. The transformants were grown on LB + ampicillin plates at 37°C overnight. We performed plasmid miniprep analysis using restriction enzymes AatII and NotI to determine orientation. We obtained one transformant in the + orientation, named PLA_2 3.8 BG341(+). We performed a large scale plasmid preparation of this DNA to obtain supercoiled plasmid.

EXAMPLE 10

Expression Of Phospholipase A_2 In COS-7 African Green Monkey Kidney Cells

We performed DNA mediated transfection using the DEAE/dextran method (L. M. Sompayrac and K. J. Danna, "Efficient Infection of Monkey Kidney Cells With DNA of Simian Virus 40" Proc. Natl. Acad. Sci. USA, 78, pp. 7575-78 (1981)). We transfected 100 mm^2 tissue culture dishes containing approximately 2 x 10^6 COS-7 cells (ATCC No. CRL 1651) with 3 ml each of 10 µg/ml of supercoiled plasmid from the following constructs: PLA_2 6.2 BG368 3(+), PLA_2 6.2 BG368 8(-), PLA_2 3.8 BG341 (+) and BG341 (as a control). We transfected five plates with each construct and then pooled the media from the five plates 72 hours after transfection. Cells from 2 plates were harvested by scraping into 150 µl of 0.36 N H_2SO_4. The plates were then rinsed with 2 x 150 µl of 0.18 N H_2SO_4 containing 150 mm NaCl and the washes combined with the cell suspension. The cell suspensions were centrifuged at 2,000 xg for 5 minutes at 4°C and the supernatant recovered.

Phospholipase A_2 activity was assayed in cell extracts (50 µl aliquots) and media (5 µl) using the ^{3}H-oleic acid labelled E.coli assay described in Example 3. We found that cells transfected with PLA_2 constructs in the + orientation relative to the adenovirus late promoter and, in particular, the media therefrom, contained significant amounts of phospholipase A_2 activity when compared to untransfected cells and cells transfected with vector alone.

The table below depicts a comparison of the levels of expression of phospholipase A_2 in COS-7 cells transfected with various PLA_2 constructs of this invention as well as controls.

TRANSIENT EXPRESSION
OF INFLAMMATORY PLA_2

Conditioned Media	PLA_2 (ng/ml)
COS cells	<0.03
COS cells transfected with BG 341 (vector control)	<0.02
COS cells transfected with PLA_2 6.2 BG368 3(+)	1
COS cells transfected with PLA_2 3.8 BG341(+)	3

The expression and secretion of phospholipase A_2 in cells transfected with PLA_2 3.8 BG341(+) indicated that in the presence of an exogenous promoter, such as the adenovirus major late promoter, the amino acids encoded by nucleotides 2453-2492 can serve as a functional signal sequence.

EXAMPLE 11

Construction Of Cell Lines That Stably Express Phospholipase A_2

Because the transfected COS-7 cells described in Example 10 expressed phospholipase A_2 only transiently, we next constructed cell lines that stably expressed the polypeptide. We employed the commercially available expression host CHO $DHFR^-$, the dihydrofolate reductase deletion mutant of the Chinese Hamster Ovary cell line.

We transfected these cells with a mixture of PLA_2 3.8 BG341(+) which had been linearized by digestion with restriction enzyme XmnI and pAdD26 (R. J. Kaufman and P. A. Sharp, "Amplification and Expression of Sequences Cotransfected With a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol., 159, pp. 661-21 (1982)) that had been linearized with restriction enzyme StuI in a 9:1 molar ratio to provide the maximum number of PLA_2

gene copies per transfectant. pAdD26 is a plasmid which contains a functional DHFR gene. Transfection was achieved by $CaPO_4$ precipitation. Alternatively transfection may be effected by electroporation or spheroplast fusion.

Following transfection, we incubated the cells for 2 days in nonselective medium α^+MEM. We then split the cells by diluting 1:10 into selective medium α^-MEM + 10% dialyzed fetal calf serum. Clones were visible after 9 days. After 11 days, cloning rings were inserted on the plates and each clone was trypsinized and transferred to one well of a 48 well microtiter plate. When clones became nearly confluent, we removed the media and assayed it for phospholipase A_2 activity. The cells were expanded in 6 well microtiter plates. We assayed 66 clones and froze in liquid nitrogen the twenty that expressed phospholipase A_2 at the highest level. The five highest expressors were subcloned for amplification in 30 nM methotrexate. These clone or others may be further amplified by growth in higher concentrations of methotrexate.

We also created a second construct, PLA_2 3.8 JODS (Figure 15B), for the expression of phospholipase A_2 in animal cells. In this vector, the phospholipase A_2 coding sequences and the DHFR coding sequences are on the same plasmid. Parent plasmid pJODS (Figure 15A) was digested with AatII and NotI. PLA_2 3.8 BG341(+) was also digested with the same enzymes. Both digests were purified on a low melt agarose gel and the appropriate band excised from the gel. The AatII-NotI fragment from PLA_2 3.8 BG341(+) (containing the promoter elements from BG341 as well as the 3.8 kb PLA_2 insert) was ligated to the AatII-NotI fragment of pJODS (containing the DHFR sequence). We then used the ligated DNA to transform E.coli MC1061 cells. Following mini prep

analysis to determine the presence of inserts, we performed large scale plasmid preparation from one clone containing the appropriate plasmid. We named the plasmid PLA_2 3.8 JODS. We then used this plasmid to transform E.coli JA221 cells and prepared CsCl-banded plasmid DNA for use in animal cell transfection. Prior to transfection, PLA_2 3.8 JODS was linearized with AatII. We routinely used 20 µg of linearized plasmid to transfect CHO DHFR$^-$ cells. Transfections, growth of cells and phospholipase A_2 assays were performed as described above.

EXAMPLE 12

Transfection Of Animal Cells With PLA_2 Genomic Clones

The bacteriophage PLA_2 8.5 EMBL3 contained approximately 16 kb of human DNA, including the PLA_2 insert which encodes mature inflammatory PLA_2. This phage is modified by standard techniques so that it contains a selectable marker for animal cell expression, such as herpes simplex virus thymidine kinase. Phage particle transfection of mouse L tk$^-$ cells is accomplished by the methods of M. Ishiura et al., "Phage Particle-Mediated Gene Transfer to Cultured Mammalian Cells", Mol. Cell Biol., 2, pp. 607-16 (1982).

Specifically, 24 hours prior to transfection, L tk$^-$ cells are plated in 100 mm^2 tissue culture dishes at a density of 5×10^5 - 1×10^6 cells per plate. For each plate, 1 ml of 3×10^7 pfu/ml phage particles are coprecipitated with calcium phosphate, pH 6.85, at 25°C for 10 minutes. The precipitate is then absorbed on the L cells for 24 hours at 37°C in 5% CO_2 in air. The cells are then washed with Hepes buffered saline, re-fed with α-MEM and grown for 40 hours. The media is then replaced with HAT media and the cells maintained

until clones appear (about 10 days). Tk^+ clones are assayed for phospholipase A_2 activity.

Alternatively, DNA mediated transfection can be carried out. DNA is prepared from either PLA_2 8.5 EMBL3 or *PSQ 130*. In the latter case, the 6.2 kb PLA_2 insert is isolated and purified by digestion with HindIII and preparative gel electrophoresis. In the former case, PLA_2 8.5 EMBL3 DNA is linearized by digestion with SalI. Each DNA preparation may then used together with an appropriate selectable marker (e.g., DHFR for CHO $DHFR^-$ cells, thymidine kinase for L tk^- cells) to cotransfect animal cells. DNA-mediated transfection is accomplished by calcium phosphate precipitation or electroporation (G. Chu et al., "Electroporation for the Efficient Transfection of Mammalian Cells With DNA", Nucl. Acids Res., 15, pp. 1311-25 (1987)). Clones positive for the selectable marker are assayed for PLA_2 activity.

EXAMPLE 13

Size Determination Of Phospholipase A_2 mRNA From Transformed COS-7 Cells

Cells from three tissue culture dishes (100 mm^2) of COS-7 cells transfected with 10 µg/ml of supercoiled plasmid from either PLA_2 6.2 BG368 3(+), PLA_2 6.2 BG368 8(-), PLA_2 3.8 BG341 (+) or BG341 (as a control) were used to prepare total RNA. The transfected cells were lysed in guanidinium isothiocyanate buffer and total RNA prepared by the method of J. M. Chirgwin et al., "Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease", Biochemistry, 18, pp. 5294-99 (1979). We analyzed 1 µg and 10 µg aliquots of total RNA from each transfected cell pool by the Northern blotting technique (H. Lehrach et al., Biochemistry, 10, pp. 4743-51 (1977)) using GeneScreen filters (New England Nuclear, MA) and following the

manufacturer's instructions. The transferred RNA was hybridized to a ^{32}P-labelled 1.4 kb OxanI fragment of PSQ 130, which had been labelled by the random priming technique (A. P. Feinberg and B. Vogelstein, "A Technique for Radiolabeling DNA Restriction Endonuclease Fragments", Anal. Biochem., 132, pp. 6-13 (1983); Ibid, "Addendum", Anal. Biochem., 137, pp. 266-67 (1984)). OxanI is the equivalent isoschizomer of commercially available restriction enzymes MstII and Bsu36. All of these enzymes recognize the nucleotide sequence: CCTNAGG. These enzymes cut PSQ 130 at nucleotides 2054-2060 and 3413-3419.

The probe hybridized to an 1100 nucleotide mRNA in cells transfected with PLA_2 3.8 BG341(+). This size is consistent with expected transcription from the adenovirus late promoter and predicted splicing and polyadenylation.

EXAMPLE 14

Synthesis Of Human Inflammatory Phospholipase A_2 cDNA

Total RNA isolated from cell lines transfected with PLA_2 3.8 BG341(+) or any other PLA_2 genomic clone which expresses human inflammatory phospholipase A_2 by the above method may be used to obtain a PLA_2 cDNA. Poly A^+ RNA is isolated from total RNA using oligo dT cellulose chromatography. The poly A+ RNA (5-10 µg) is resuspended in H_2O at a concentration of 5 µg/µl and treated with 2.5 mM CH_3HgOH at room temperature for 10 minutes. β-mercaptoethanol is then added to a final concentration of 0.035 M. Synthesis of cDNA is carried out using a cDNA synthesis kit (BRL; catalogue #8267SA) according to manufacturer's directions. The double stranded cDNA is then ligated to linker 35-36:

5'AATTCGAGCTCGAGCGCGGCCGC3'

3' GCTCGAGCACGCGCCGGCG5'

using standard procedures. The cDNA is then size selected using Select 4L, 5L, or 6L columns (5 Prime-3 Prime Inc., West Chester, PA) according to manufacturer's directions. The size-selected cDNA is then ligated to EcoRI digested λgt10. Aliquots of the ligation reaction are packaged in Gigapack (Stratagene, San Diego, CA) according to the manufacturer's protocol. The packaged phage are then used to infect E.coli BNN102 cells and plated for amplification. PLA_2 cDNA clones are then obtained by screening the resultant library with antisense oligonucleotides from the PLA_2 coding region of PLA_2 3.8 BG341(+).

Alternatively, PLA_2-specific mRNA is enriched initially by hybrid selection with PLA_2 genomic DNA using any one of the methods described in R. Jagus, "Hybrid Selection of mRNA and Hybrid Arrest of Translation" Meth. Enzymol., 152, pp. 567-72 (1987).

The predicted nucleotide sequence of the PLA_2 coding region of the above-described cDNA is:

AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA
GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC
CAC TGT GGC GTG GGT GGC AGA GGA TCC CCC AAG GAT GCA
ACG GAT CGC TGC TGT GTC ACT CAT GAC TGT TGC TAC AAA
CGT CTG GAG AAA CGT GGA TGT GGC ACC AAA TTT CTG AGC
TAC AAG TTT AGC AAC TCG GGG AGC AGA ATC ACC TGT GCA
AAA CAG GAC TCC TGC AGA AGT CAA CTG TGT GAG TGT GAT
AAG GCT GCT GCC ACC TGT TTT GCT AGA AAC AAG ACG ACC
TAC AAT AAA AAG TAC CAG TAC TAT TCC AAT AAA CAC TGC
AGA GGG AGC ACC CCT CGT TGC.

Microorganisms and recombinant DNA molecules prepared by the processes of this invention are exemplified by cultures deposited in the In Vitro International, Inc. culture collection, in

Linthicum, Maryland. These include cultures deposited on July 11, 1988 and identified as:

PSQ130/E.coli MC1061

PLA_2 6.2 BG368 3(+)/E.coli MC1061

PLA_2 3.8 BG341(+)/E.coli MC1061

PLA_2 3.8 JODS/E.coli JA221;

and cultures deposited on July 12, 1988 and identified as:

PLA_2 8.5 EMBL3/E.coli LE392.

These deposits were assigned accession numbers IVI 10174-10178, respectively.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constuctions can be altered to provide other embodiments which utilize the processes, polypeptides and DNA sequences of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by specific embodiments which have been presented hereinbefore by way of example.

CLAIMS

We Claim:

1. A process for purifying an acid stable phospholipase A_2 from a biological source comprising the steps of:

a) extracting said source with an acid;

b) replacing the acid in the extract produced in step a) with a buffer suitable for cation exchange chromatography;

c) contacting said extract with a cation exchange resin to bind the phospholipase A_2 contained in the extract;

d) eluting said phospholipase A_2 from said resin;

e) concentrating said phospholipase A_2;

f) separating said phospholipase A_2 from contaminants by molecular sizing chromatography; and

g) separating said phospholipase A_2 from contaminants remaining after step f) by reverse phase HPLC.

2. The process according to claim 1, further comprising the steps of electrophoresing said phospholipase A_2 and transferring said electrophoresed phospholipase A_2 to a solid support.

3. The process according to claim 2, wherein said electrophoresing is effected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and said solid support is a polyvinylidene difluoride membrane.

4. The process according to claim 1 or 2, wherein said phospholipase A_2 is located within a cell.

5. The process according to claim 4, further comprising before step a), the step of releasing said phospholipase A_2 from said cell.

6. The process according to claim 5, wherein said biological source comprises human, non-pancreatic cells.

7. The process according to claim 4, wherein said biological source comprises platelets.

8. The process according to claim 1 or 2, wherein said phospholipase A_2 is located extracellularly.

9. The process according to claim 8, wherein said biological source comprises human extracellular fluid.

10. The process according to claim 9, wherein said biological source comprises rheumatoid synovial fluid.

11. The process according to claim 1 or 2, wherein said acid is sulfuric acid having a pH of about 1.

12. A polypeptide comprising the amino acid sequence:

Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu.

13. A polypeptide consisting essentially of at least five consecutive amino acids selected from the amino acid sequence:

Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu.

14. The polypeptide according to claim 13, wherein said polypeptide has the sequence:

H_2N-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-COOH.

15. An oligonucleotide comprising a nucleotide sequence selected from the group consisting of nucleotide sequences which code for the polypeptide according to claim 12 or 13.

16. The oligonucleotide according to claim 15, selected from the group consisting of PLA_2-06, PLA_2-07, PLA_2-08, PLA_2-09, PLA_2-10, PLA_2-11, PLA_2-12, PLA_2-13, and combinations thereof.

17. Phospholipase A_2 purified by the process according to claim 1 or 2.

18. A pharmaceutical composition for eliciting antibodies to acid-stable inflammatory phospholipase A_2 comprising an immunologically effective amount of an immunogen which comprises a polypeptide according to claim 13 coupled to a carrier.

19. A pharmaceutical composition effective as an anti-inflammatory agent comprising a therapeutically effective amount of an antibody selected from the group consisting of antibodies to acid stable inflammatory phospholipase A_2, antibodies to the polypeptide according to claim 12, and combinations thereof.

20. A composition comprising an amount of an antibody selected from the group consisting of antibodies to acid stable inflammatory phospholipase A_2, antibodies to the polypeptide according to claim 12, and combinations thereof, wherein said

composition is effective to detect the presence of acid stable inflammatory phospholipase A_2 in a biological sample.

21. A method for treating inflammation comprising the step of treating a patient in a pharmaceutically effective manner with a composition according to claim 19.

22. A method for detecting the presence of acid stable inflammatory phospholipase A_2 in a biological sample comprising the step of contacting said sample with a composition according to claim 20.

23. The use of a pharmaceutically effective amount of an antibody to human inflammatory phospholipase A_2 for the treatment of inflammation in mammals.

24. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A_2-like polypeptide, said DNA sequence comprising the sequence:

C CAT GGG AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC.

25. A process for producing a human inflammatory phospholipase A_2-like polypeptide comprising the step of culturing a unicellular host transformed with a recombinant DNA molecule according to claim 24.

26. The recombinant DNA molecule according to claim 24, wherein said DNA molecule is PSQ 130.

27. A DNA sequence coding for a human inflammatory phospholipase A_2-like polypeptide, said DNA sequence being selected from the group consisting of:

(a) the 6.2 kb PLA_2 inserts of PLA_2 8.5 EMBL3, PSQ 130, and PLA2 6.2 BG368 3(+),

(b) the 3.8 kb PLA_2 inserts of PLA_2 3.8 BG341(+) and PLA_2 3.8 JODS,

(c) DNA sequences which hybridize to the foregoing PLA_2 inserts and which code on expression for a human inflammatory phospholipase A_2-like polypeptide, and

(d) DNA sequences which code on expression for a human inflammatory phospholipase A_2-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

28. The DNA sequence according to claim 27, said DNA sequence being selected from the group consisting of a DNA sequence with the formula:

(a) AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC CCC AAG GAT GCA ACG GAT CGC TGC TGT GTC ACT CAT GAC TGT TGC TAC AAA CGT CTG GAG AAA CGT GGA TGT GGC ACC AAA TTT CTG AGC TAC AAG TTT AGC AAC TCG GGG AGC AGA ATC ACC TGT GCA AAA CAG GAC TCC TGC AGA AGT CAA CTG TGT GAG TGT GAT AAG GCT GCT GCC ACC TGT TTT GCT AGA AAC AAG ACG ACC TAC AAT AAA AAG TAC CAG TAC TAT TCC AAT AAA CAC TGC AGA GGG AGC ACC CCT CGT TGC.

29. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A_2-like polypeptide, said DNA sequence being selected from the group consisting of:

(a) the 6.2 kb PLA_2 insert of PLA_2 8.5 EMBL3,

(b) DNA sequences which hybridize to the foregoing DNA insert and which code on expression for a human inflammatory phospholipase A_2-like polypeptide; and

(c) DNA sequences which code on expression for a human inflammatory phospholipase A_2-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

30. The recombinant DNA molecule according to claim 29, wherein said DNA sequence is the 3.8 kb PLA_2 insert of PSQ 130.

31. The recombinant DNA molecule according to claim 29, said molecule being selected from the group consisting of: PLA_2 8.5 EMBL3, PLA_2 6.2 BG368 3(+), PLA_2 3.8 BG341(+) and PLA_2 3.8 JODS.

32. A host transformed with the recombinant DNA molecule according to claim 29, wherein said host is selected from the group consisting of animal cells, insect cells, plant cells, yeast cells and other fungal cells.

33. The host according to claim 32, selected from the group consisting of COS-7 cells and CHO $DHFR^-$ cells.

34. A process for producing a human inflammatory phospholipase A_2-like polypeptide comprising the step of culturing the host according to claim 32.

35. A human inflammatory phospholipase A_2-like polypeptide produced by the process according to claim 34.

36. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A_2-like polypeptide, said DNA sequence being selected from the group consisting of:

(a) AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC CCC AAG GAT GCA ACG GAT CGC TGC TGT GTC ACT CAT GAC TGT TGC TAC AAA CGT CTG GAG AAA CGT GGA TGT GGC ACC AAA TTT CTG AGC TAC AAG TTT AGC AAC TCG GGG AGC AGA ATC ACC TGT GCA AAA CAG GAC TCC TGC AGA AGT CAA CTG TGT GAG TGT GAT AAG GCT GCT GCC ACC TGT TTT GCT AGA AAC AAG ACG ACC TAC AAT AAA AAG TAC CAG TAC TAT TCC AAT AAA CAC TGC AGA GGG AGC ACC CCT CGT TGC,

(b) DNA sequences which hybridize to the foregoing DNA sequence and which code on expression for a human inflammatory phospholipase A_2-like polypeptide; and

(c) DNA sequences which code on expression for a human inflammatory phospholipase A_2-like polypeptide coded for on expression by any of the foregoing DNA sequences.

37. A host transformed with the recombinant DNA molecule according to claim 36, said host being selected from the group consisting of animal cells, plant cells, yeast and other fungi, and bacteria.

38. A process for producing a human inflammatory phospholipase A_2-like polypeptide comprising the step of culturing the host according to claim 37.

39. A human inflammatory phospholipase A_2-like polypeptide produced by the process according to claim 38.

40. The recombinant DNA molecule according to any one of claims 24, 29 or 36, said molecule further comprising an expression control sequence, said expression control sequence being operatively linked to said DNA sequence coding for a human inflammatory phospholipase A_2-like polypeptide.

41. A process for producing a human inflammatory phospholipase A_2-like polypeptide comprising the steps of:

(a) transfecting a eukaryotic host with a DNA sequence comprising the 6.2 kb PLA_2 insert; and

(b) culturing said transfected host.

42. The process according to claim 41, wherein said eukaryotic host is selected from the goup consisting of a mouse L-cell and a CHO DHFR cell.

43. A human inflammatory phospholipase A_2-like polypeptide produced by the process according to claim 41.

44. The human phospholipase A_2-like polypeptide according to any one of claims 35, 39 or 43, said polypeptide being selected a polypeptide of the formula:

NLVNFHRMIK LTTGKEAALS YGFYGCHCGV GGRGSPKDAT DRCCVTHDCC YKRLEKRGCG TKFLSYKFSN SGSRITCAKQ DSCRSQLCEC DKAAATCFAR NKTTYNKKYQ YYSNKHCRGS TPRC.

45. A polypeptide coded for on expression by a DNA sequence selected from the group consisting of the DNA sequences of claim 27 or 28, said polypeptide being essentially free of other proteins of human origin.

FIGURE 1

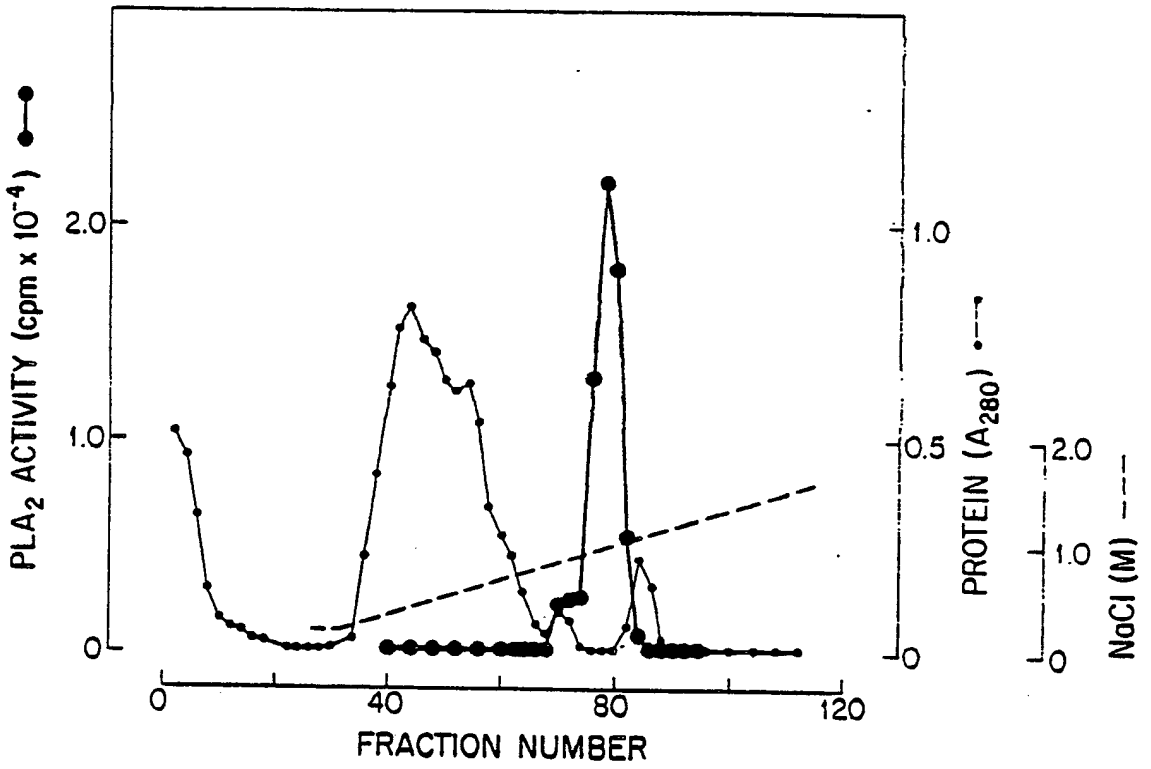

PLA2 ACTIVITY (cpm x 10-4)
2.0
1.0
0
1.0
0.5
0
PROTEIN (A280)
2.0
1.0
0
NaCl (M)
0
40
80
120
FRACTION NUMBER

FIGURE 2

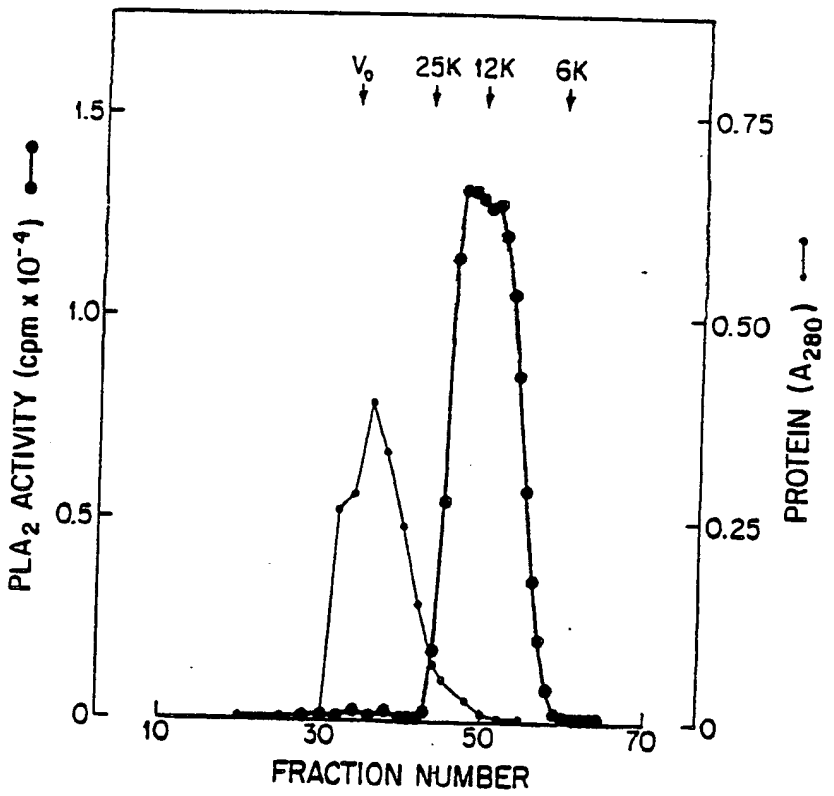

V_o
25K
12K
6K
1.5
1.0
0.5
0
0.75
0.50
0.25
0
PLA_2 ACTIVITY (cpm x 10^{-4})
PROTEIN (A_{280})
10
30
50
70
FRACTION NUMBER

FIGURE 3

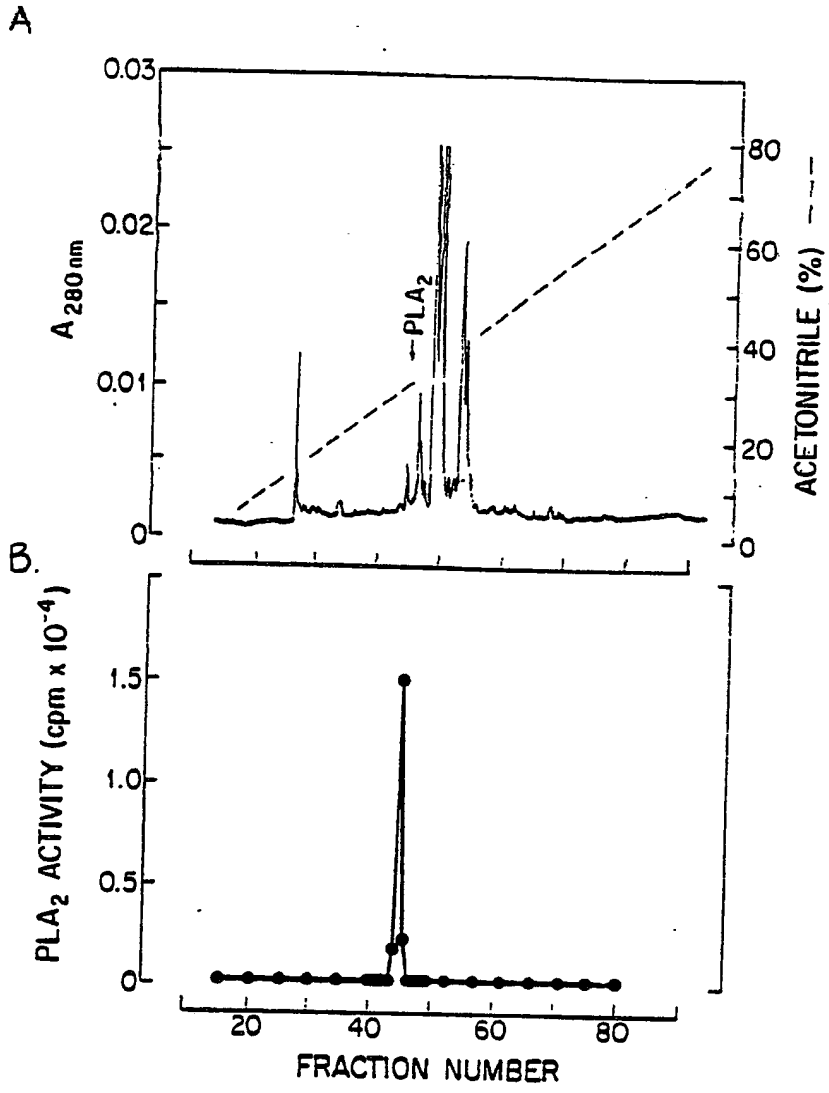

A
0.03
0.02
0.01
0
$A_{280\,nm}$
PLA_2
80
60
40
20
0
ACETONITRILE (%)
B.
PLA_2 ACTIVITY (cpm x 10^{-4})
1.5
1.0
0.5
0
20
40
60
80
FRACTION NUMBER

FIGURE 4

STEP	PROTEIN MG	PLA_2 ACTIVITY TOTAL UNITS	PLA_2 ACTIVITY UNITS/MG	RECOVERY %	PURIFICATION -FOLD
SONICATION	7510	16	0.0021		
EXTRACTION (PH 1)	6060	26	0.0043		
DIALYSIS (PH 4.5) **	718	1001	1.41	100	11
FAST S CHROMATOGRAPHY	2.5	413	165	41	1300
G-50 GEL FILTRATION	0.1	961	9440	96	>740,000
REVERSE-PHASE HPLC	<0.01	341	146,750	34	>1,100,000

FIGURE 5

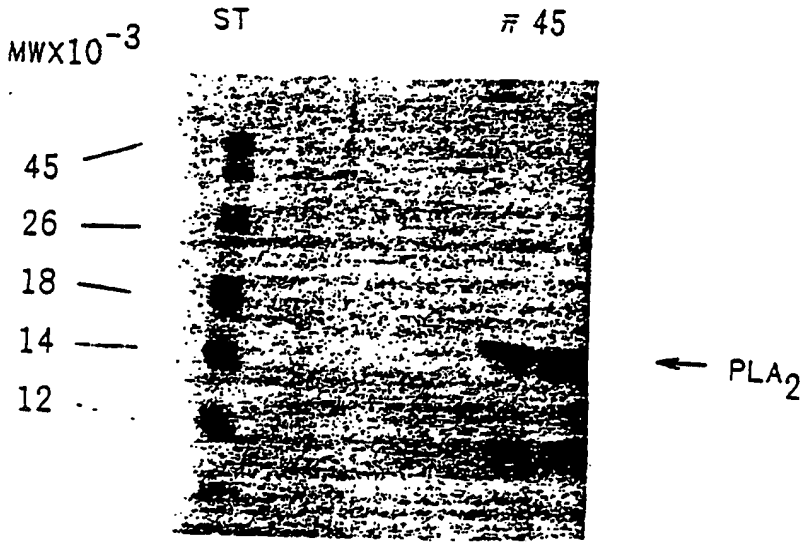

COOMASSIE-STAINED PVDF-MEMBRANE
MWX10-3
ST
45
45
26
18
14
12
PLA2

FIGURE 6

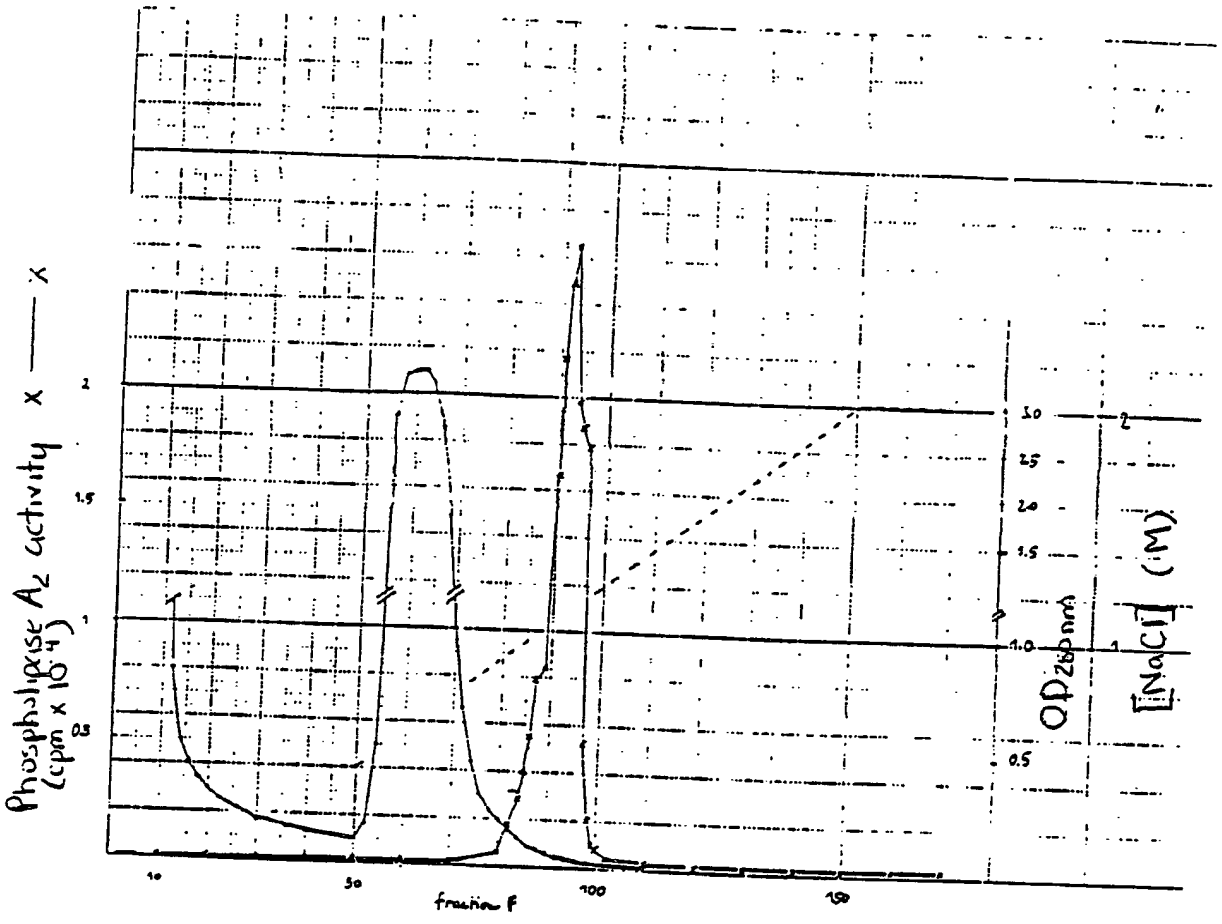

Phospholipase A2 activity x — x
(cpm x 10^-4)
2
1.5
1
0.5
10
50
100
150
fraction F
3.0
2.5
2.0
1.5
1.0
0.5
OD280nm
2
1
[NaCl] (M)

FIGURE 7

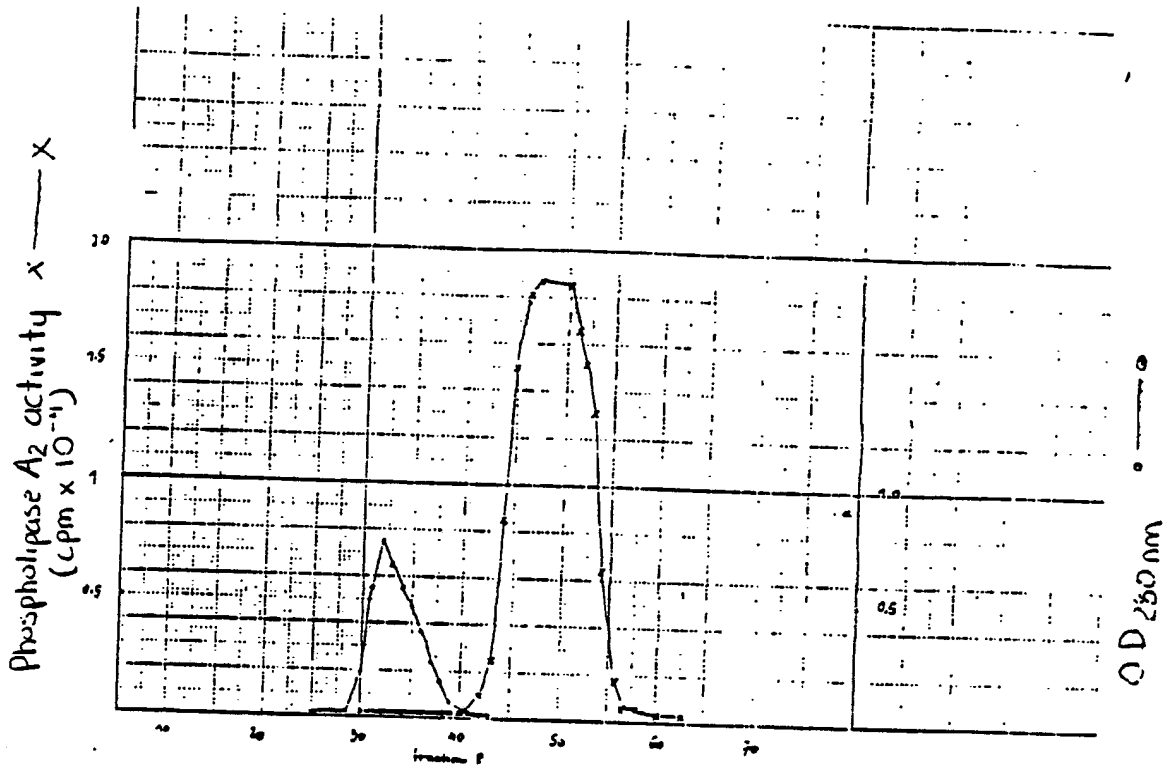

Phospholipase A_2 activity x——x
(cpm x 10^{-4})
2.0
1.5
1
0.5
1.0
0.5
O D 280 nm

FIGURE 8

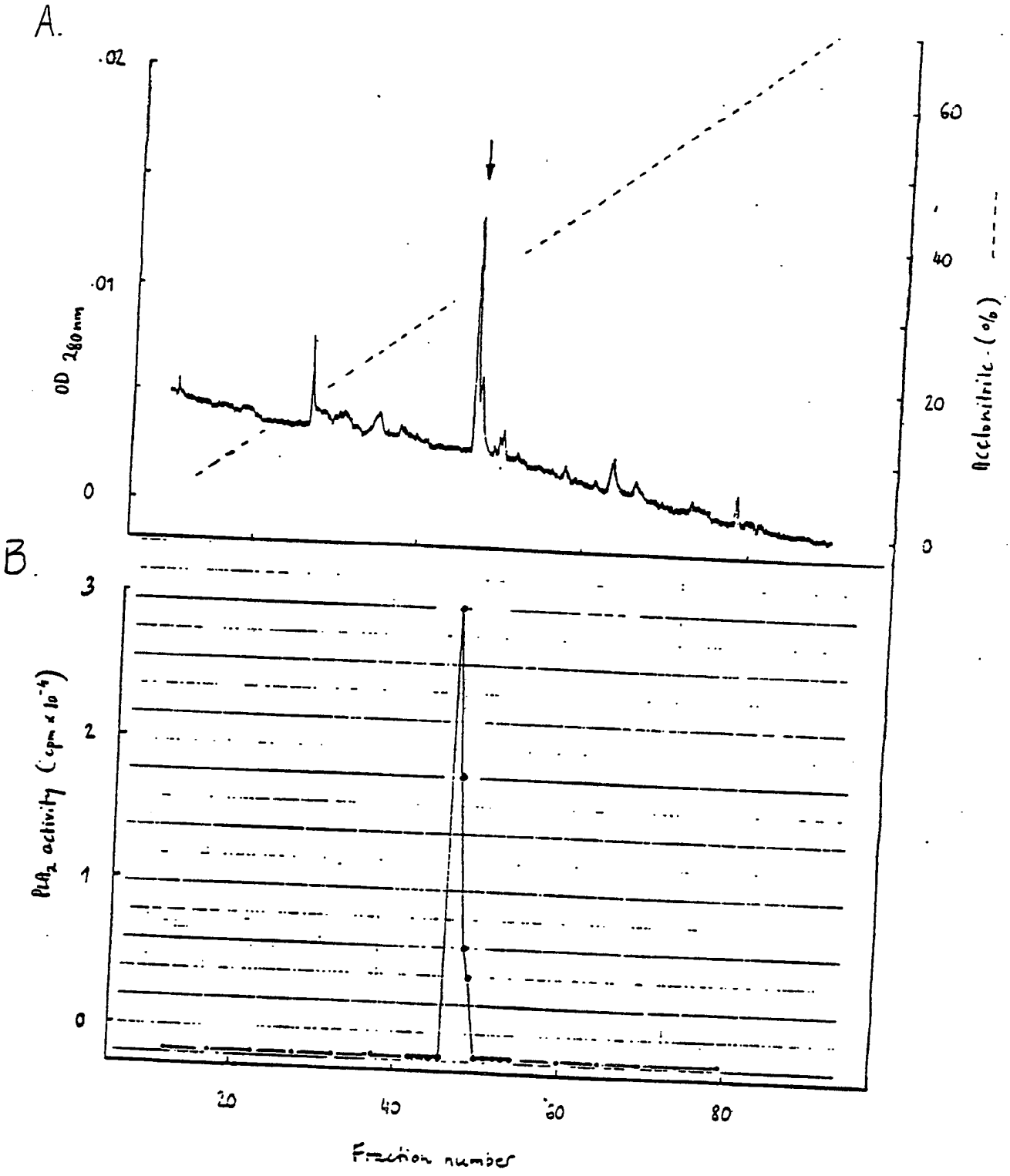
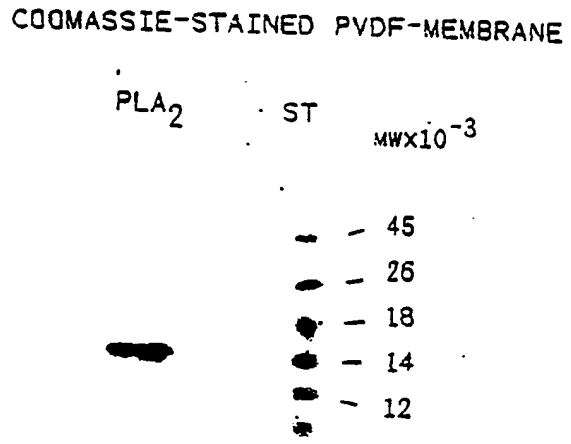

A.
.02
.01
0
OD 280nm
60
40
20
0
Acetonitrile (%)
B.
3
2
1
0
PLA2 activity (cpm x 10^-4)
20
40
60
80
Fraction number

FIGURE 9

PURIFICATION OF HUMAN RHEUMATOID SYNOVIAL FLUID PLA_2

STEP	PROTEIN MG	PLA_2 ACTIVITY TOTAL UNITS	PLA_2 ACTIVITY UNITS/MG	RECOVERY %	PURIFICATION -FOLD
H_2SO_4-EXTRACTION	1932	4.89	0.003	100	
DIALYSIS (PH 4.5)	1582	4.80	0.003	98	
FAST S CHROMATOGRAPHY	4.2	0.36	0.085	7	
G-50 GEL FILTRATION	<0.1	2.03	20.3	42	>8,000
REVERSE-PHASE HPLC	<0.01	2.81	280.7	57	>100,000

FIGURE 10


COOMASSIE-STAINED PVDF-MEMBRANE
PLA2
ST
MWx10-3
45
26
18
14
12

11/23

Figure 11 A

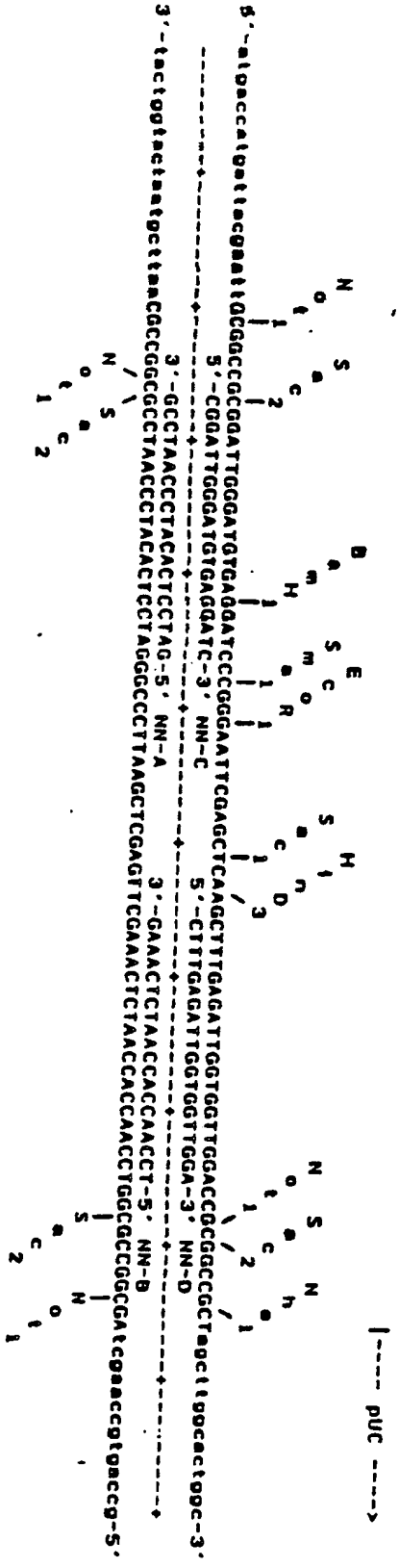


Figure 11B

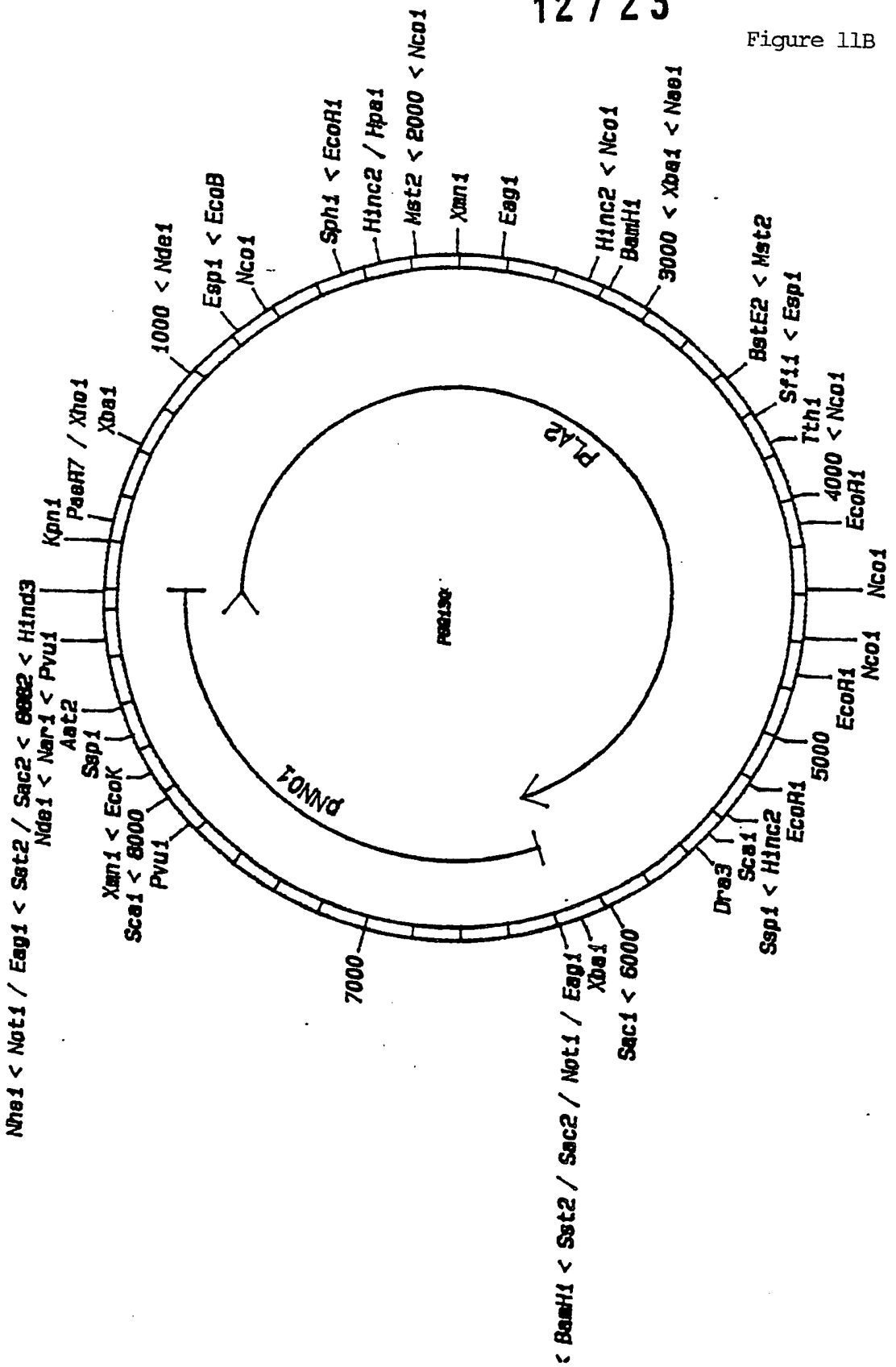
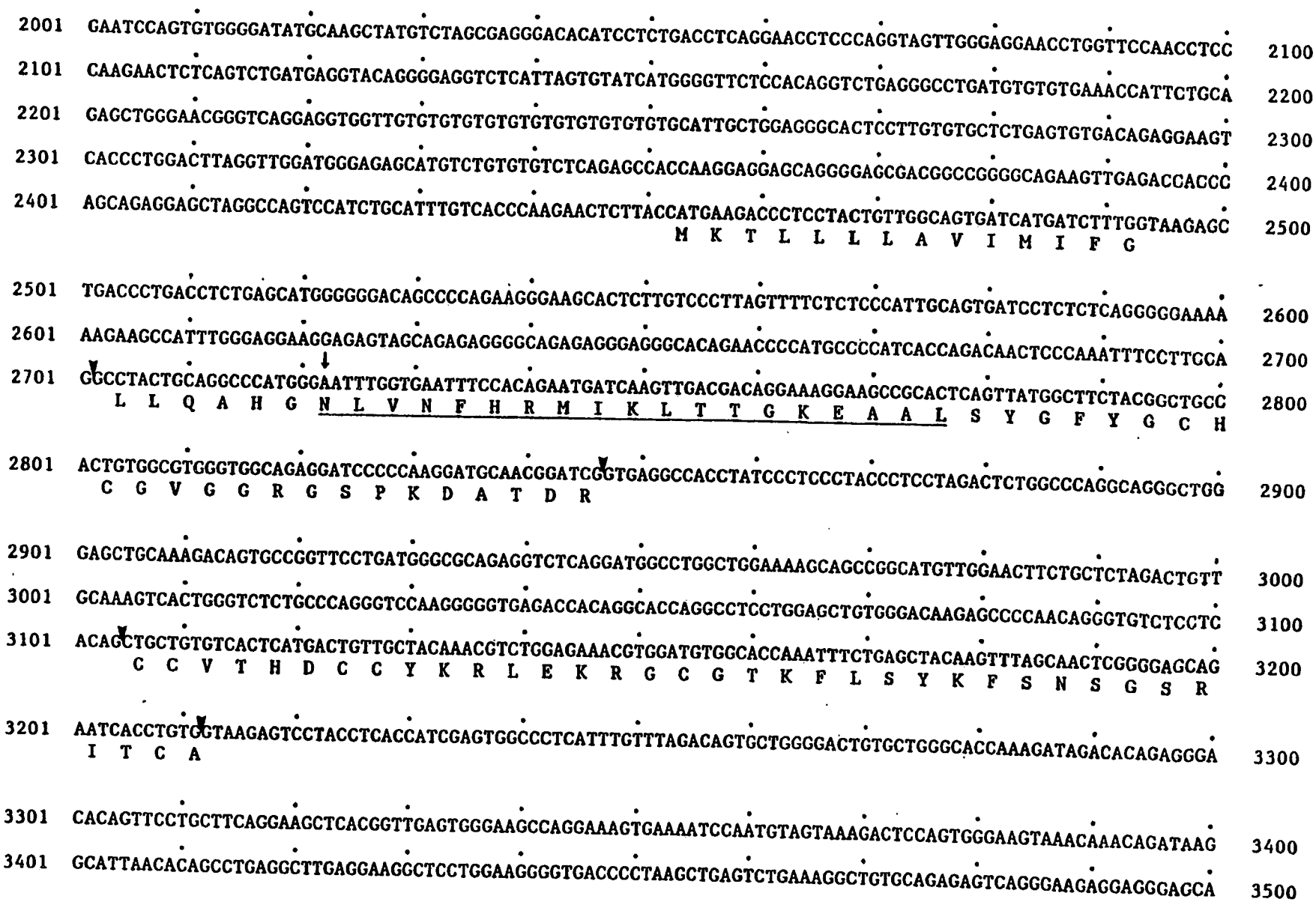

pLA2
pUN01
Nde1 < Nar1 < Pvu1
Aat2
Ssp1
EcoK
Xmn1 < 8000
Sca1 < Pvu1
Nhe1 < Not1 / Eag1 < Sst2 / Sac2 < Hind3
Kpn1
PaeR7 / Xho1
Xba1
1000 < Nde1
Esp1 < EcoB
Nco1
Sph1 < EcoR1
Hinc2 / Hpa1
Mst2 < 2000 < Nco1
Xmn1
Eag1
Hinc2 < Nco1
BamH1
3000 < Xba1 < Nae1
BstE2 < Mst2
Sf11 < Esp1
Tth1
4000 < Nco1
EcoR1
Nco1
Nco1
EcoR1
5000
EcoR1
Hinc2
Sca1
Dra3
Ssp1 < Hinc2
Sac1 < 6000
Xba1
Eag1
< BamH1 < Sst2 / Sac2 / Not1 / Eag1
7000

Figure 12

```
1     AAGCTTCACCCTGATCTCTGCCTTCATCTTTGTATGGCCTTCTCCCAGTGTGCCTGCCTGTCTCCAAATTTCCCATTTTGTAATGACATCTGTCATCTT  100
101   GGATTAGGGCCCCATCCTAGCTGCCCTAGTGATCTCCTCTTAACTAATTATATCTGCAATGACCCTATTTTTAAATAAGATCACATTCTAAGGTACCGGGA  200
201   CTCCAACACAGGAACTGAGAGCAGGAAGGAGGACCACAGTCCAACCCCTTACACAGTCGAGCACTGGAGGTAAATGGTATTCTCGAGAGGAAAGCTGTTT  300
301   GATGGCTCTTGGGATAGATTAGGTTATGCTCCAGCAACTGCCCATCTCAATTATCATCAGCTTAATACAACAAAAGTCTATTTCTCACTCACACTAAGAC  400
401   CCCTGTGAGCTGCAGGACTTTCTAGGACAACTGTCTTCCGTGCCATGATTCTACACCCCAGATTGCTTTCATCTTCTGGTTCTACCAGTGGGGCAGGGAA  500
501   GGGCTGGGGAGTTGCATCTCATAGTCCCCTTGCTGGAGAAACCTGGAAGTGACCCTTCATACCAGTGCTCATGTTTTACCAGTCAGCAGGCAGGGCCCTAG  600
601   GTCTCCATCCCCTGCAAGGGGCTCTAGAGTGTAGGGAACAGATGGAATGTTCACACAAGCACCACAGCCCCTGCCACAGTGACTTTTTTAGGACTGGTATCGC  700
701   AGAGTGTTTACTTAAGGCGGTGGAAGCTAAATTCTTAGCATGTGCTGGAGAGGCAATGAAAAAGATATTTACTTTATGAATTAAAGCTGGAGTCAGTGTCA  800
801   GCCCGAAGGCTGAAGGAAAAAGAGCAACAGATCCAGGGAGCATTCACCTGCCCTGTCTCCAAACAGGTGAGGATGGGGAATAAAGTGAAGGCAGTGCTTT  900
901   GGTGGGAACTTCAAGGATAGCCTCTGGCTTTTTCCAGGTTTAGAAGCTCATATGAGACAGGGGTGGAGGAAAAGAAGAAGAAGAATAAGAAGAGAAAGT  1000
1001  TGAGGCCCTGGCCCAAGTTAGTGGGAAGGGAAATCCACACCCCCATTAAACTCTCTCCCTGGTGGACTGTGTGGGTGCACACGTGAGGCCTGCACAGGTGCTG  1100
1101  GAACATGGTAGAGGCCCAGGACATACTTCCTGTGAATGATTGAGCGGCTGAATGAATGAGTACCACTAAAAGCCCTCTTTTCTATTCCCAAATGCC  1200
1201  ACATTGAGCAGAAGGAGCAATCCTTGCTCAGCAATTGGTAGTCCCTTTGGGTGTGCAAATGAGTCCACACAGCCTGCAACACAGCAGTGTCTGTGCCCCCC  1300
1301  TTAGAGGCCGATTGCAGGGAGGTGGCTGACTGTTGATCACACCCAGAGCTGGTTATGGGAATTTACTCCATGGAAAGACTGCAAACTGCCTGAAATGTGT  1400
1401  TTTGGCATCAGCTACTGACACGTAAGGTTTCCCAATCCTCAACTCTGTCCTGCCAGGCTGATGAGGGGAAGGAAGGGATTACCTAGGGGTATGGGCGACC  1500
1501  AATCCTGAGTCCACCAACTGACCACGCCCATCCCCAGCCTTGTGCCTCACCTACCCCCAACCTCCCAGAGGGAGCAGCTATTTAAGGGGAGGCAGGAGTGC  1600
1601  AGAACAAACAAGACGGCCTGGGGATACAACTCTGGAGTCCTCTGAGAGGTAAAGAGCCAGCGAAGCTGATGTCCTGTCAAGAGCAGAATTCCTGCTCATT  1700
1701  CGCTGCCTTTGAGAGTGGCTGTGTTGTGCATGCATGTGCATTTGATATGTATGAGAGGGTGTGTGTGCATGAGTGTGTTGAGTGAGTATGTGAGTGT  1800
1801  AGTGTAAGAGAGGATGTTGGCACTATCAGGTAATTACGAGAGTGTGTATGTGGCCATAGGTGTGTTAACATGTATGTGTTTGGGAACTTGTGTATGTG  1900
1901  GAAGGGGTTAGAAGGCCTAGAAGAGAGAGGTTGATGCTTTCATTCTGGAGGAAAATACTGAGGCCGAGCCTCCATGGGTGCCTTGGAGACTCCAAGCCTT  2000
```


2001 GAATCCAGTGTGGGGATATGCAAGCTATGTCTAGCGAGGGACACATCCTCTGACCTCAGGAACCTCCCAGGTAGTTGGGAGGAACCTGGTTCCAACCTCC 2100
2101 CAAGAACTCTCAGTCTGATGAGGTACAGGGGAGGTCTCATTAGTGTATCATGGGGTTCTCCACAGGTCTGAGGGCCTGATGTGTGTGAAACCATTCTGCA 2200
2201 GAGCTGGGAACGGGTCAGGAGGTGGTTGTGTGTGTGTGTGTGTGTGTGTGTGTGCATTGCTGGAGGGCACTCCTTGTGTGCTCTGAGTGTGACAGAGGAAGT 2300
2301 CACCCTGGACTTAGGTTGGATGGGAGAGCATGTCTGTGTGTCTCAGAGCCACCAAGGAGGAGCAGGGGAGCGACGGCCGGGGCAGAAGTTGAGACCACCC 2400
2401 AGCAGAGGAGCTAGGCCAGTCCATCTGCATTTGTCACCCAAGAACTCTTACCATGAAGACCCTCCTACTGTTGGCAGTGATCATGATCTTTGGTAAGAGC 2500
M K T L L L L A V I M I F G
2501 TGACCCTGACCTCTGAGCATGGGGGGACAGCCCCAGAAGGGAAGCACTCTTGTCCCTTAGTTTTCTCTCCCATTGCAGTGATCCTCTCTCAGGGGGAAAA 2600
2601 AAGAAGCCATTTGGGAGGAAGGAGAGTAGCAGAGAGGGGCAGAGAGGGAGGGCACAGAACCCCATGCCCCATCACCAGACAACTCCCAAATTTCCTTGCA 2700
2701 GGCCTACTGCAGGCCCATGGGAATTTGGTGAATTTCCACAGAATGATCAAGTTGACGACAGGAAAGGAAGCCGCACTCAGTTATGGCTTCTACGGCTGCC 2800
L L Q A H G N L V N F H R M I K L T T G K E A A L S Y G F Y G C H
2801 ACTGTGGCGTGGGTGGCAGAGGATCCCCCAAGGATGCAACGGATCGGTGAGGCCACCTATCCCTCCCTACCCTCCTAGACTCTGGCCCAGGCAGGGCTGG 2900
C G V G G R G S P K D A T D R
2901 GAGCTGCAAAGACAGTGCCGGTTCCTGATGGGCGCAGAGGTCTCAGGATGGCCTGGCTGGAAAAGCAGCCGGCATGTTGGAACTTCTGCTCTAGACTGTT 3000
3001 GCAAAGTCACTGGGTCTCTGCCCAGGGTCCAAGGGGGTGAGACCACAGGCACCAGGCCTCCTGGAGCTGTGGGACAAGAGCCCCAACAGGGTGTCTCCTC 3100
3101 ACAGCTGCTGTGTCACTCATGACTGTTGCTACAAACGTCTGGAGAAACGTGGATGTGGCACCAAATTTCTGAGCTACAAGTTTAGCAACTCGGGGAGCAG 3200
C C V T H D C C Y K R L E K R G C G T K F L S Y K F S N S G S R
3201 AATCACCTGTGGTAAGAGTCCTACCTCACCATCGAGTGGCCCTCATTTGTTTAGACAGTGCTGGGGACTGTGCTGGGCACCAAAGATAGACACAGAGGGA 3300
I T C A
3301 CACAGTTCCTGCTTCAGGAAGCTCACGGTTGAGTGGGAAGCCAGGAAAGTGAAAATCCAATGTAGTAAAGACTCCAGTGGGAAGTAAACAAACAGATAAG 3400
3401 GCATTAACACAGCCTGAGGCTTGAGGAAGGCTCCTGGAAGGGGTGACCCCTAAGCTGAGTCTGAAAGGCTGTGCAGAGAGTCAGGGAAGAGGAGGGAGCA 3500

```
3501  TTCCCAGAAGAGGACACAGCATGGTCAAAGGCACTAAAGGGCACTGTAAGCCATTCTGTACTGCCCAGCAGAAACATGAGGAAGAGGAGCAGTGCTGAGC  3600
3601  CATGATGCTGGAGACATAGGAAGGAGCTAGGTCAATCCGGCCCTCCAGGCCGGGCTGTATTTAGGTTTTGCCCTAAAGCAATAGGATGCTATTAAGCAAA  3700
3701  GGAGCTACAGGGTCAGATTTGCATTTTAGATGACTCACTGTGGGGACAGGGTCGATGGAGACAAGTGGAAGGGGGGCAGAGAAAGCTATTGCCATCATGC  3800
3801  AGGCAAGAGGGAGTAACATCTTGACATAAAACAATGGAGGTCAGGATGGGAAAGGTGGAGAAAAAATCAAGATGCATTTGAGATGGAATCAGCTGAACTG  3900
3901  GTGACTGAGTTGGGAGGGATGGGGAGAGGGAGTTGTTGGATGGATATGTGGCTGCATGGATGGCACAACTGTGATAAAGACCATGGGAGCAGGTCATGGT  4000
4001  GGAGGGTGGGGAGGAGCAGTTCTATTTCCAGCATGTTGAGTTTAGGGGCCTCCAGCACCCAGGGAGGGGTCCAGCAGGCAGCTGTCTATACAAATGCAGC  4100
4101  TCAGGGGAGAATTCAGGACTGGGACACAGATTCAGAAGCCAGCAGCAGAGACCTGAGAGGTGGGTGTGATCACTCATTTGCTGTTTAAAGGCCCAGAAAG  4200
4201  GAGACAGAGAAGGGATGGACAGAGAGGGAGAAGGGGAACTGAGCGAGAAGGTCAAGGAGTCAGTAAGGAAATGGTTAGCAAGGGCCAAGTGAACAGGGAG  4300
4301  TCCTCCATGAAAAGGGCCAACAAGGCTCCCCTGGATGTTGAGGCAGAAACGCATGAGGGACTCAGGGGAAGCTGTTTCCATGGAGTCGGGAGGGCAAAGC  4400
4401  CAGATTAGACCAGGTGGGGGCTGATGGGAAGGCAAATAAAGACAGGAGGCAAAGACAACATTCTGGAGAAAGTTTGGCCCTGAAGGGGAGGAGAGGTGGG  4500
4501  TGGCACTGGAAGGCTTTGCTTGGTGTCCCCAGACAGCTGACTCATGAGTGGGATTTGGAAAAAGCGTGGACTCCTGCCCATGGCCTGAGTCCTTTAAGAT  4600
4601  CAGAAATTATGTCTCCCATCATGGCCTCTCCATAGAGGCATGTATCTTCAGCAGGCGTTAGGTCACAAGCCACATGATGCCAAGCTGACAGTGGCTTGCA  4700
4701  TAATGGGGATATGTGACTGTCGCATAACTAGAATTCTGGAAGAGTGCAGTGCCAGGCTTGGGGCAGCTTTCCAGCCATGTCATTAAGAATCCAGCCTTCT  4800
4801  CCTGGCCTTCAGCTATGCCACGTGGCCAGTGTCTACACCTGGGATGTCAAGAGACAGGCTGCAGGTCCACCCTCCTGGCCTCATACTATGGAAGAGGCTT  4900
4901  TCCTTTTGGGCATCTCTCTTTTTGGAGGGAGGAAATAGATCGTTCCCAGAAGCCCCCAGCAGACTTCCCCTTGTTGCTCATTGGTTGGAACAAGGTTACA  5000
5001  TGATACACAAAGACCAATCACTGCAAAGGAAAAAGGGATGACCCTGCCTGGCTTACACCAATCACAATCTATTCCCAGACCCCCGAGGCTAGGGCTTTGC  5100
5101  CTCCTGGACACATCTGTTAGCAAGAGGAAGAGATTATGGCTGTTAGGAAGGCCTTTGAGAAAGTATCCCAGTGCCTGGCTGTGTCTCCACCAGGCTGGAG  5200
5201  GCCAGCATCCCAAGGGCAAGAATTCTGTCTCCCCATTGGTCAGAAATATCTGGAGCGCAGGTGTTTGTCTCCAACTAGGAGCTTCTGGAGGACAGGGCTG  5300
5301  TGTCTTCTACCCTAGGGTTCCCACAAGAAGCCACTGAATATTAATAAACTCCCATCTTGTGTTTATTTTCTTATGATTTCAGCAAAACAGGACTCCTGCA  5400
                                                                                                  K  Q  D  S  C  R
```

```
5401  GAAGTCAACTGTGTGAGTGTGATAAGGCTGCTGCCACCTGTTTTGCTAGAAACAAGACGACCTACAATAAAAAGTACCAGTACTATTCCAATAAACACTG  5500
        S  Q  L  C  E  C  D  K  A  A  A  T  C  F  A  R  N  K  T  T  Y  N  K  K  Y  Q  Y  Y  S  N  K  H  C

5501  CAGAGGGAGCACCCCTCGTTGCTGAGTCCCCTCTTCCCTGGAAACCTTCCACCCAGTGCTGAATTTCCCTCTCTCATACCCTCCCTCCCTACCCTAACCA  5600
        R  G  S  T  P  R  C  *

5601  AGTTCCTTGGCCATGCAGAAAGCATCCCTCACCCATCCTAGAGGCCAGGCAGGAGCCCTTCTATACCCACCCAGAATGAGACATCCAGCAGATTTCCAGC  5700
5701  CTTCTACTGCTCTCCTCCACCTCAACTCCGTGCTTAACCAAAGAAGCTGTACTCCGGGGGGTCTCTTCTGAATAAAGCAATTAGCAAATCATGTATATGT  5800
5801  GTGTGTGTATGCATTGCCCTATGCACTGTTAGCATGAGAACAAACAGGGGTAGGGGTTAAAAATACAGGCTCTGAGTCTGGTGTAGTGCTGTGTGCCTGT  5900
5901  TGTCCCAGCTGCTCGGGAGGCTGAGGCTGAAGTGCAAGGATTGCTTGAGCTCACAGAAGTTCAAGGCCAGCCTGGGCAACACAGGGAGACCTTATCTTTA  6000
6001  TAAAAACAAAACAAAACAAACGAAAAAAATAGCTTCTGGAGTCAGACTGGCTGTGTCAAATCCCCGCTCCCCCATCTCACTAAGCCTTAGTTCTTTCATC  6100
6101  TAGATGGAGATAACACTGGTGCCTCCTGCATGGGCATGTTTGAGAATTAGAATGATGACAAATGAAAAGCTT  6172
```

```
                                                                    1
Group I   (Bovine)      ALWQFNGMIK CKIPSSEPLL DFNNYGCYCG LGGSGTPVDD LDRCCQTHDN
Group II  (C. atrox)    SLVQFETLIM KIAGRSGLLW .YSAYGCYCG WGGHGLPQDA TDRCCFVHDC
6.2 kb PLA2 Insert      NLVNFHRMIK LTTGKEAALS .YGFYGCHCG VGGRGSPKDA TDRCCVTHDC
Consensus               -L--F---I- ----------L- ----YGC-CG -GG-G-P-D- -DRCC--HD-

                                                                   51
Group I   (Bovine)      CYKQAKKLDS CKVLVDNPYT NNYSYSCSNN EITCSSENNA CEAFICNCDR
Group II  (C. atrox)    CYG...KATD CNPKTV...S YTY.SEENGE II.C.GGDDP CGTQICECDK
6.2 kb PLA2 Insert      CYKRLEKRG. CGTKFL.... .SYKFSNSGS RITC.AKQDS CRSQLCECDK
Consensus               CY----K--- C--------- --Y------- -I-C------ C----C-CD-

                                                                  101
Group I   (Bovine)      NAAICFSKVP YNKEHKNLDK K.N.C..... ..
Group II  (C. atrox)    AAAICFRDNI PSYDNKYWLF PPKDCREEPE PC
6.2 kb PLA2 Insert      AAATCFARNK TTYNKKYQYY SNKHCRGSTP RC
Consensus               -AA-CF---- -----K---- ----C----- --
                                                                  132
```

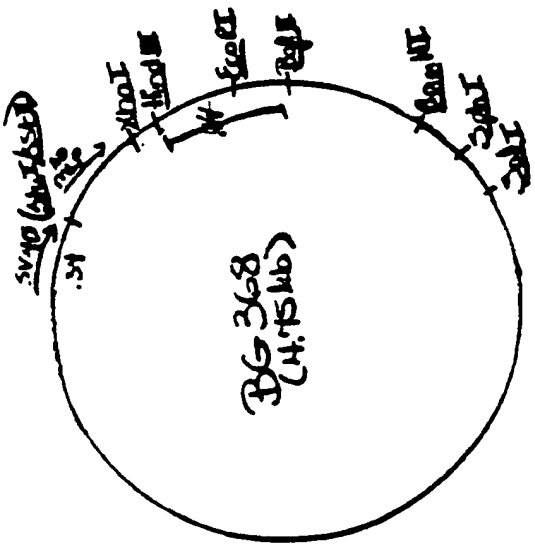
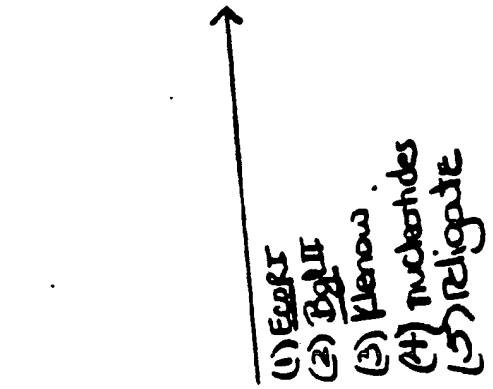

pG 368
(4.15 kb)

Figure 14A


(1) EcoRI
(2) BglII
(3) Klenow
(4) nucleotides
(5) ligate

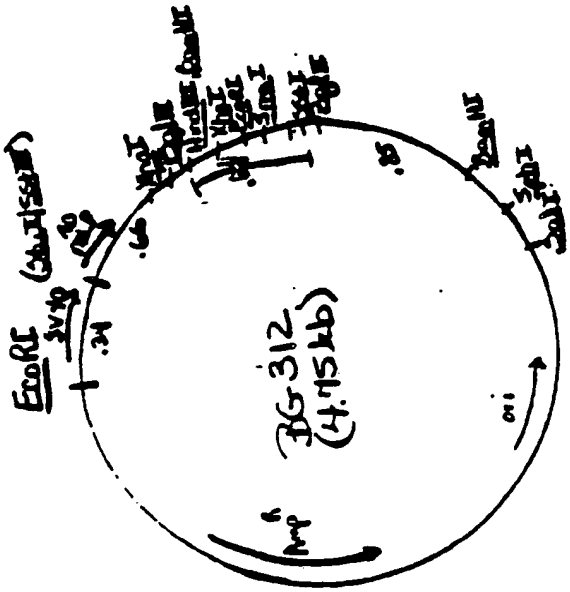

pG 312
(4.15 kb)

Figure 14B

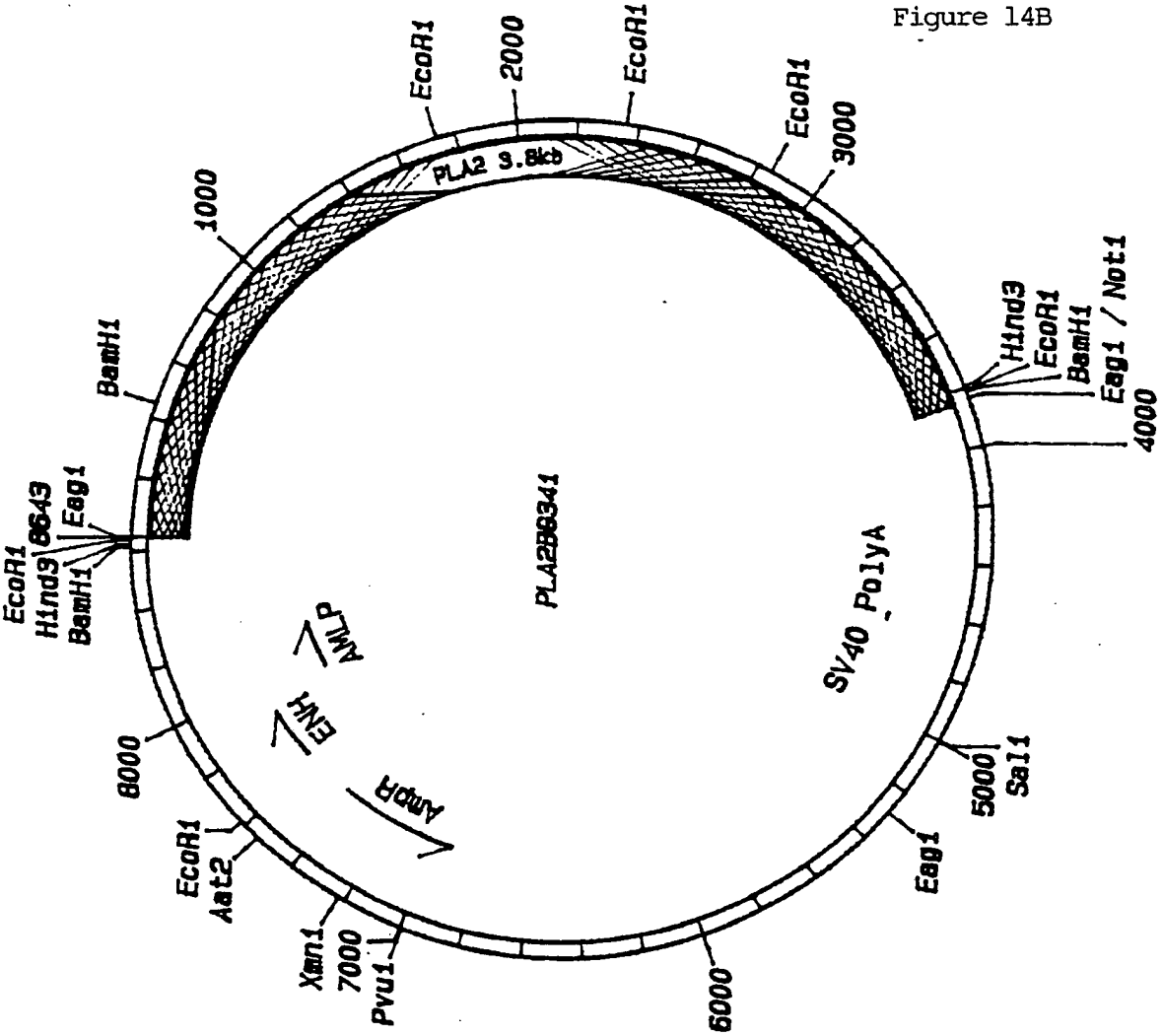

EcoR1
2000
EcoR1
EcoR1
3000
PLA2 9.8kb
1000
BamH1
Hind3
EcoR1
BamH1
Eag1 / Not1
4000
8643
Eag1
EcoR1
Hind3
BamH1
PLA2 8643
AMLP
ENH
AmpR
SV40 PolyA
8000
5000
Sal1
EcoR1
Aat2
Eag1
Xmn1
7000
Pvu1
6000

Figure 14C

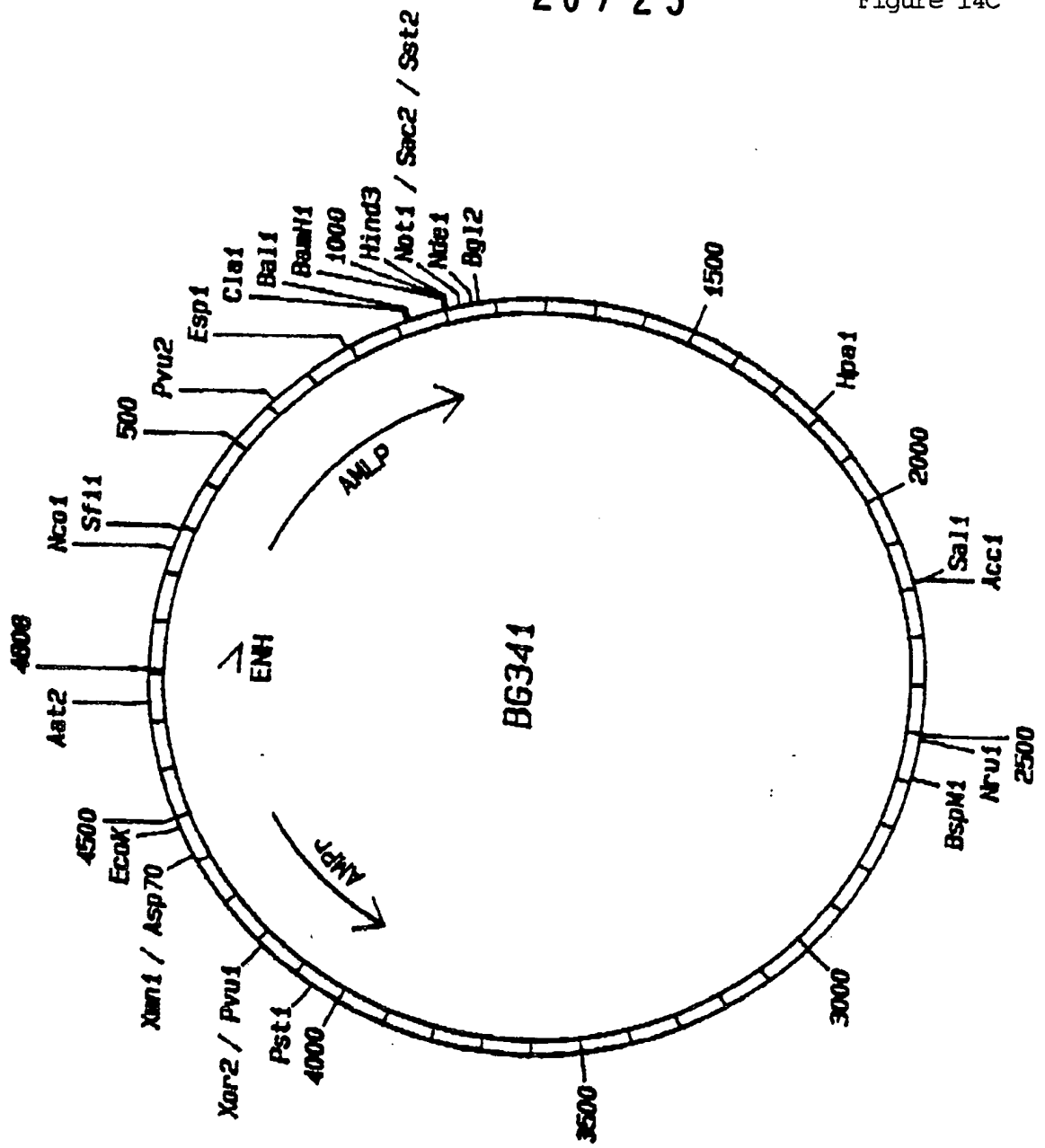

Sac2 / Sst2
Not1
Nde1
Bgl2
Hind3
1000
BamH1
Bal1
Cla1
Esp1
Pvu2
500
Sfi1
Nco1
4000
Aat2
4500
EcoK
Asp70
Xmn1 / Pvu1
Pst1
4000
Xor2 / Pvu1
3500
3000
Nru1
2500
BspM1
Acc1
Sal1
2000
Hpa1
1500
AMLo
AMPr
ENH
BG341

Figure 14 D

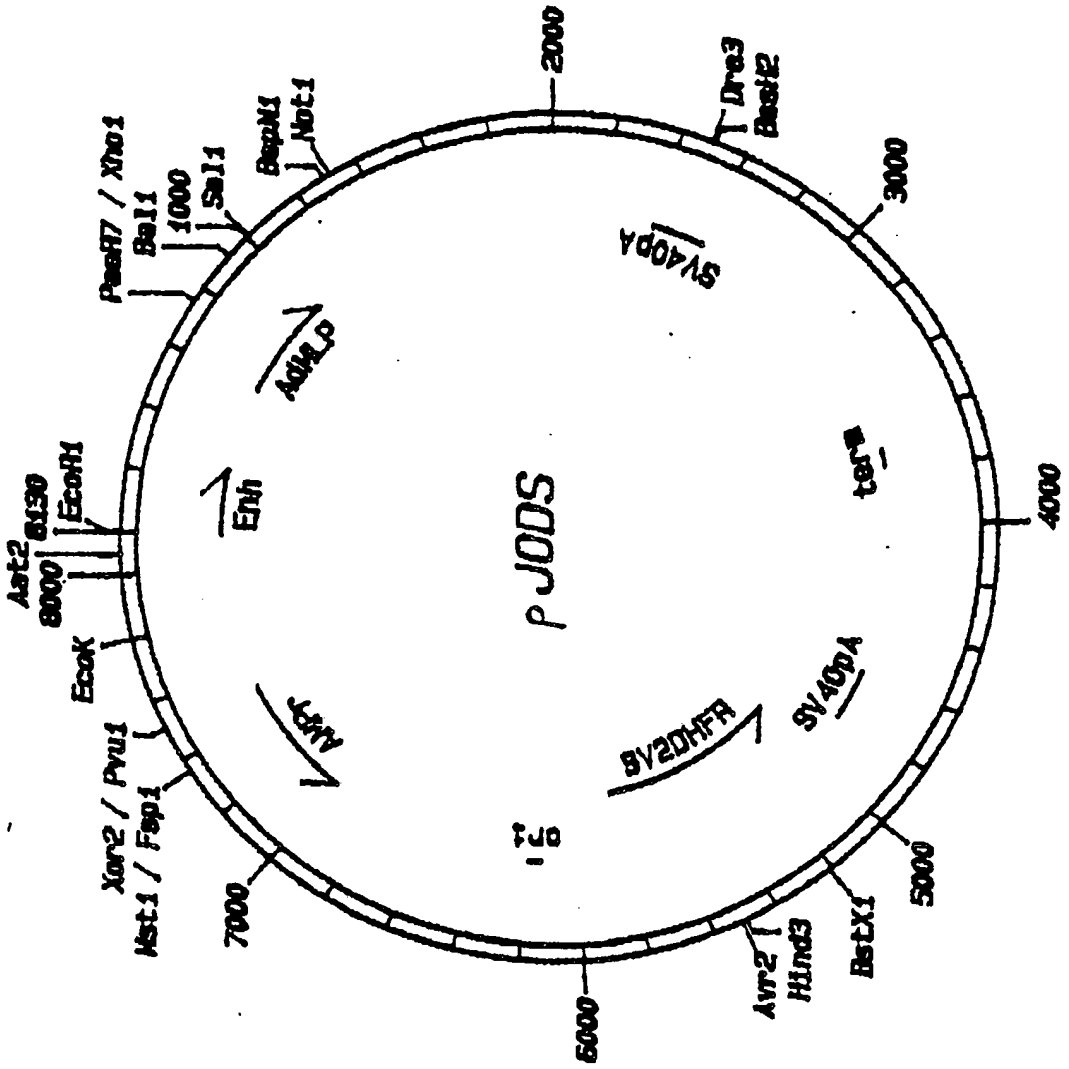

pJODS
2000
3000
4000
5000
6000
7000
1000
Dra3
BssH2
BspM1
Not1
Sal1
Pasi7 / Xho1
Bal1
SV40pA
AdMLP
Enh
EcoH1
Ast2
6130
8000
EcoK
Xor2 / Pvu1
Nst1 / Fsp1
AMP
SV2DHFR
SV40pA
ter
ori
Avr2
Hind3
BstX1

Figure 15 A

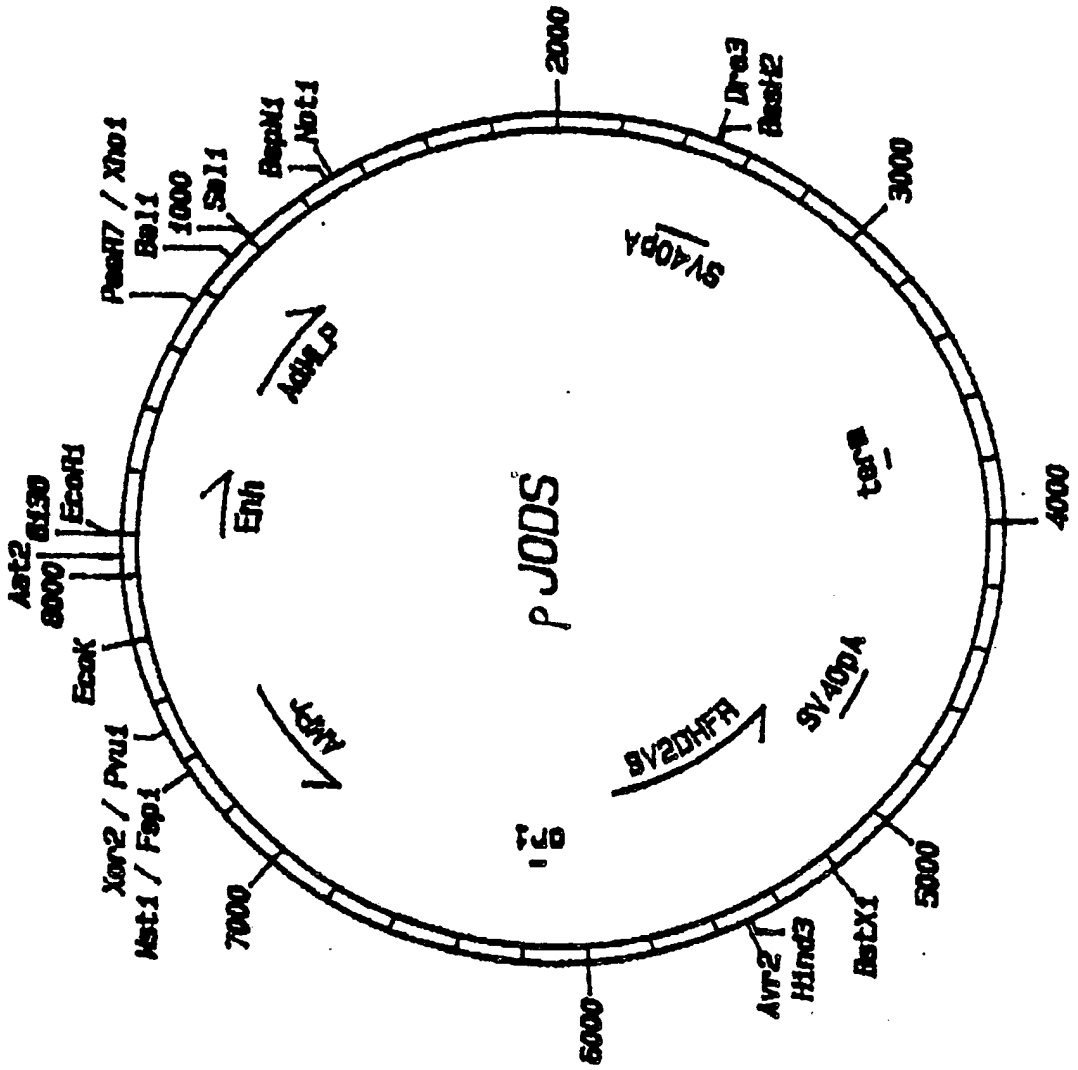
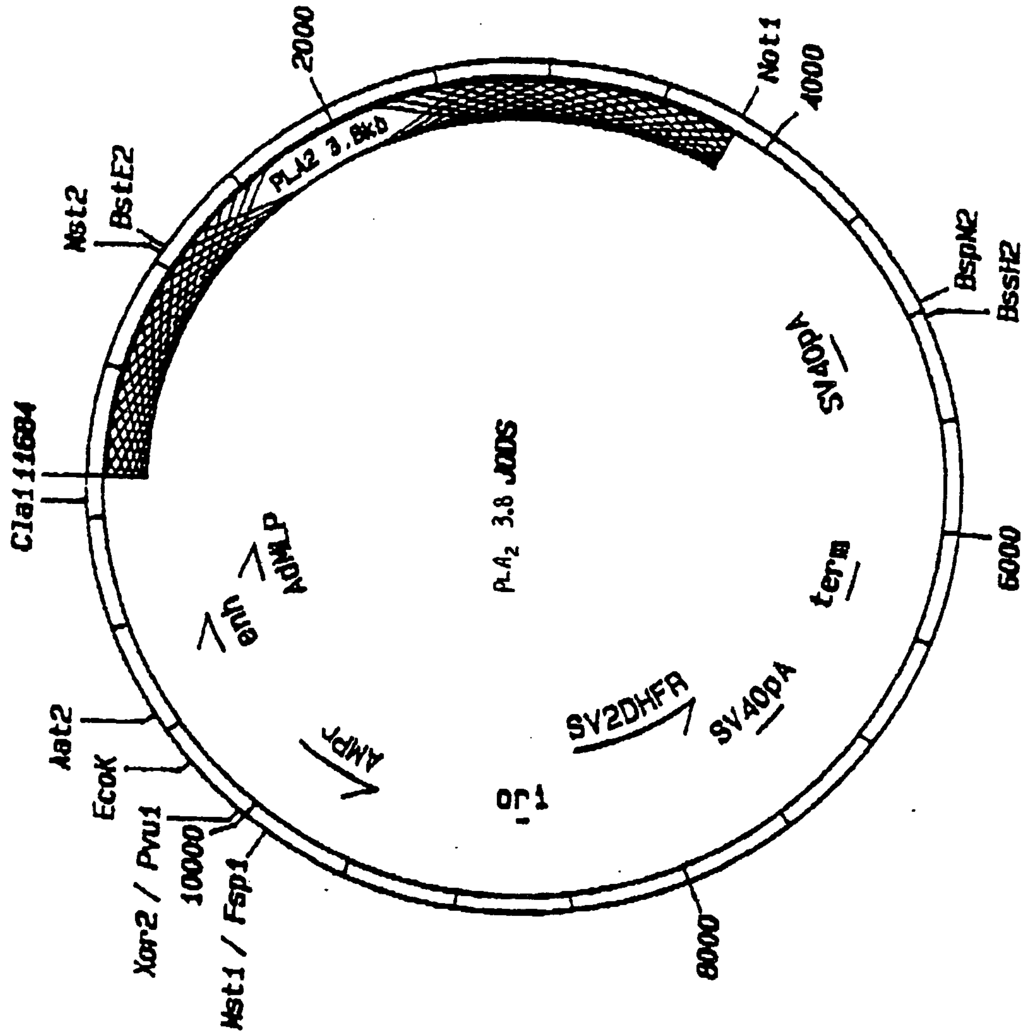

pJODS
2000
3000
4000
5000
6000
7000
1000
Dra3
BssH2
SV40pA
ter
SV40pA
SV2DHFR
AMP
Xor2 / Pvu1
Nst1 / Fsp1
Ast2
8000
EcoK
8139
EcoR1
Enh
AdMLP
PaeR7 / Xho1
Bg11
Sal1
BspM1
Not1
Avr2
Hind3
BstX1

Figure 15B


2000
Not1
4000
BspM2
BssH2
6000
8000
Mst1 / Fsp1
10000
Xor2 / Pvu1
EcoK
Aat2
Cla1 11684
Mst2
BstE2
PLA2 3.8kb
SV40pA
term
SV2DHFR
SV40pA
ori
AMPr
enh
AdMLP

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/01418

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 9/16, C 07 K 7/08, A 61 K 37/02, A 61 K 39/392, C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N, C 12 Q, A 61 K, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, vol. 105, no. 23, 8 December 1986, (Columbus, Ohio, US), R.M. Kramer et al.: "Solubilization and properties of calcium-dependent human platelet phospholipase A2", see page 262, abstract no. 205215g, & Biochim. Biophys. Acta 1986, 878(3), 394-403	17
Y	--	1-11
Y	J. Biochem., vol. 101, no. 5, 1987, M. Hayakawa et al.: "Amino acid composition and NH_2-terminal amino acid sequence of rat platelet secretory phospholipase A_21", pages 1311-1314 see the whole article cited in the application	1-11
	--	
Y	J. Biochem., vol. 102, no. 1, 1987, H.W. Chang et al.: " Purification	1-11
	./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16th August 1989	13 SEP 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	and characterization of extracellular phospholipase A_2 from peritoneal cavity of caseinate-treated rat", pages 147-154 see the whole article cited in the application	
Y	The Journal of Biological Chemistry, vol. 261, no. 23, 15 August 1986, The American Society of Biological Chemists, Inc., (US), L.A. Loeb et al.: "Identification and purification of sheep platelet phospholipase A_2 isoforms", pages 10467-10470 see the whole article cited in the application	1-11
Y	Biochemistry, vol. 25, 1986, American Chemical Society, S Forst et al.: "Structural and functional properties of a phospholipase A2 purified from an inflammatory exudate", pages 8381-8385 see the whole article cited in the application	1-11
Y	Chemical Abstracts, vol. 92, no. 9, 3 March 1980, (Columbus, Ohio, US), J. Salak et al.: "Isolation of corynebacterial enzymes from cultivation media by gel filtration and ion-exchange chromatography", see page 313, abstract no. 72356t, & Toxicon 1979, 17(6), 655-8	1-11
A	Chemical Abstracts, vol. 103, no. 11, 16 September 1985, (Columbus, Ohio, US), T.L. Hazlett et al.: "Affinity chromatography of phospholipase A_2 from Naja naja naja (Indian cobra) venom", see page 272, abstract no. 83963k, & Toxicon 1985, 23(3), 457-66	
A	Chemical Abstracts, vol. 103, no. 1, 8 July 1985, (Columbus, Ohio, US), M.M. Rakhimov et al.: "Biospecific adsorption chromatography of phos- ./.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

pholipase A2 from different sources", see page 245, abstract no. 2623z, & Prikl. Biokhim. Mikrobiol. 1985, 21(2), 190-8

--

A J. Biochem., vol. 99, no. 3, 1986, O. Ohara et al.: "Dog and rat pancreatic phospholipases A2: Complete amino acid sequences deduced from complementary DNAs", pages 733-739

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers ...21.., because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/55, 15/70, 9/18, A61K 37/64, C12N 1/21, C12P 21/08, G01N 33/573, C12Q 1/44

A1

(11) International Publication Number: **WO 95/00649**

(43) International Publication Date: 5 January 1995 (05.01.95)

(21) International Application Number: PCT/GB94/01374

(22) International Filing Date: 24 June 1994 (24.06.94)

(30) Priority Data:

9313144.9	25 June 1993 (25.06.93)	GB
9400413.2	11 January 1994 (11.01.94)	GB

(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM PLC [GB/GB]; New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MACPHEE, Colin, Houston [GB/GB]; SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR (GB). TEW, David, Graham [GB/GB]; SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR (GB). SOUTHAN, Christopher, Donald [GB/GB]; SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR (GB). HICKEY, Deirdre, Mary, Bernadette [IE/GB]; SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR (GB). GLOGER, Israel, Simon [GB/GB]; SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD (GB). LAWRENCE, Geoffrey, Mark, Prouse [GB/GB]; SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD (GB). RICE, Simon, Quentyn, John [GB/GB]; SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD (GB).

(74) Agent: VALENTINE, Jill, Barbara; SmithKline Beecham plc, Corporate Intellectual Property, SB House, Great West Road, Brentford, Middlesex TW8 9BD (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: LIPOPROTEIN ASSOCIATED PHOSPHOLIPASE A_2, INHIBITORS THEREOF AND USE OF THE SAME IN DIAGNOSIS AND THERAPY

(57) Abstract

The enzyme Lp-PLA_2 in purified form, an isolated nucleic acid molecule encoding Lp-PLA_2, the use of an inhibitor of the enzyme Lp-PLA_2 in therapy and a method of screening compounds to identify those compounds which inhibit the enzyme.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

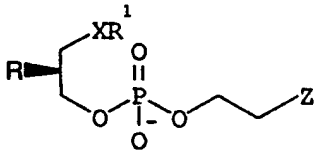
AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

LIPOPROTEIN ASSOCIATED PHOSPHOLIPASE A2, INHIBITORS THEREOF AND USE OF THE SAME IN DIAGNOSIS AND THERAPY

The present invention relates to the use of inhibitors of an enzyme in the therapy, in particular in the treatment of atherosclerosis. The present invention also relates to the isolation and purification of the enzyme, to isolated nucleic acids encoding the enzyme, to recombinant host cells transformed with DNA encoding the enzyme, to the use of the enzyme in diagnosing a patient's susceptibility to atherosclerosis, and to the use of the enzyme in identifying compounds which are potentially useful for the treatment of atherosclerosis.

Lipoprotein Associated Phospholipase A_2 (Lp-PLA_2), also previously known in the art as Platelet Activating Factor Acetyl Hydrolase (PAF acetyl hydrolase). During the conversion of LDL to its oxidised form, Lp-PLA_2 is responsible for hydrolysing the sn-2 ester of oxidatively modified phosphatidylcholine to give lyso-phosphatidylcholine and an oxidatively modified fatty acid. Both of these products of Lp-PLA_2 action are potent chemoattractants for circulating monocytes. As such, this enzyme is thought to be responsible for the accumulation of cells loaded with cholesterol ester in the arteries, causing the characteristic 'fatty streak' associated with the early stages of atherosclerosis. Inhibition of the Lp-PLA_2 enzyme would therefore be expected to stop the build up of this fatty streak (by inhibition of the formation of lysophosphatidylcholine), and so be useful in the treatment of atherosclerosis. In addition, it is proposed that Lp-PLA_2 plays a direct role in LDL oxidation. This is due to the poly unsaturated fatty acid-derived lipid peroxide products of Lp-PLA_2 action contributing to and enhancing the overall oxidative process. In keeping with this idea, Lp-PLA_2 inhibitors inhibit LDL oxidation. Lp-PLA_2 inhibitors may therefore have a general application in any disorder that involves lipid peroxidation in conjunction with the enzyme activity, for example in addition to conditions such as atherosclerosis and diabetes other conditions such as rheumatoid arthritis, stroke, myocardial infarction, reperfusion injury and acute and chronic inflammation.

The present invention therefore provides in a first aspect an inhibitor of the enzyme lipoprotein associated Lp-PLA_2 for use in therapy, in particular in the treatment of atherosclerosis. Suitable compounds able to inhibit the Lp-PLA_2 enzyme are known in the art and include for example, the following compounds of structure (I):



(I)

in which R is C_{1-6}alkylCONR^2;

R^2 is hydrogen or C_{1-6}alkyl;

X is oxygen, sulphur or -O(CO)-;

R^1 is C_{8-20}alkyl;

Z is $N(R^3)_2$, $^{\oplus}N(R^3)_3$, SR^3, $^{\oplus}S(R^3)_2$, in which each group R^3 is the same or different and is C_{1-6} alkyl, OR^2, C_{1-4}alkanoyl, imidazolyl or N-methylimidazolyl

Suitably R^2 is hydrogen or C_{1-6} alkyl; preferably R^2 is hydrogen.

Suitably X is oxygen, sulphur or -O(CO)-; preferably X is oxygen

Suitably R^1 is C_{8-20}alkyl; preferably R^1 is C_{16-18} alkyl

Suitably Z is $N(R^3)_2$, $^{\oplus}N(R^3)_3$, SR^3, $^{\oplus}S(R^3)_2$, in which each group R^3 is the same or different and is C_{1-6} alkyl, OR^2, C_{1-4}alkanoyl, imidazolyl or N-methylimidazolyl; preferably Z is SR^3 in which R^3 is methyl or OR^2 in which R^2 is hydrogen

The compounds of structure (I) can be prepared by processes known to those skilled in the art, for example as described in J Chem Soc Chem Comm.,1993, 70-72; J Org Chem, 1983, 48, 1197 and Chem Phys Lipids, 1984,35,29-37 or procedures analogous thereto.

When used in therapy, the compounds of structure (I) are formulated in accordance with standard pharmaceutical practice.

The compounds of structure (I) and their pharmaceutically acceptable salts which are active when given orally can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and, lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilised and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises a compound of formula (I) or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

Preferably the composition is in unit dose form such as a tablet or capsule.

Each dosage unit for oral administration contains preferably from 1 to 250 mg (and for parenteral administration contains preferably from 0.1 to 25 mg) of a compound of the formula (I) or a pharmaceutically acceptable salt thereof calculated as the free base.

The daily dosage regimen for an adult patient may be, for example, an oral dose of between 1 mg and 500 mg, preferably between 1 mg and 250 mg, or an intravenous, subcutaneous, or intramuscular dose of between 0.1 mg and 100 mg, preferably between 0.1 mg and 25 mg, of the compound of the formula (I) or a pharmaceutically acceptable salt thereof calculated as the free base, the compound being administered 1 to 4 times per day. Suitably the compounds will be administered for a period of continuous therapy.

The enzyme, lipoprotein associated Lp-PLA_2 has not hitherto been available in isolated purified form. The present invention therefore provides in a further aspect, the enzyme lipoprotein associated Lp-PLA_2 in purified form. By purified form is meant at least 80%, more preferably 90%, still more preferably 95% and most preferably 99% pure with respect to other protein contaminants.

The enzyme Lp-PLA_2 may be characterised by one or more partial peptide sequences selected from SEQ ID NOs:1, 2, 3, 4, 10 and 11 or by the partial peptide sequence comprising residues 271 to 441 or consisting of residues 1 to 441 of SEQ ID NO:9. The enzyme Lp-PLA_2 may further or alternatively characterised by its molecular weight found to be 45kDa, at least 45kDa, 45-47kDa, 46-47kDa or 45-50kDa.

The invention also provides fragments of the enzyme having Lp-PLA_2 activity.

The enzyme can be isolated and purified using the methods hereafter described. Once isolated, the protein sequence of the enzyme can be obtained using standard techniques. In identifying said sequence, a number of protein fragments have been identified, each of which comprises part of the whole sequence of the

enzyme. These sequences are themselves novel and form a further aspect of the invention.

This invention also provides isolated nucleic acid molecules encoding the enzyme, including mRNAs, DNAs, cDNAs as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

In particular, the invention provides an isolated nucleic acid molecule consisting of bases 1 to 1361 or 38 to 1361 or comprising the sequence corresponding to bases 848 to 1361 of SEQ ID NO: 9.

This invention also provides recombinant vectors, such as cloning and expression plasmids useful as reagents in the recombinant production of the enzyme, as well as recombinant prokaryotic and/or eukaryotic host cells comprising the novel nucleic acid sequence.

This invention also provides nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to the novel nucleic acid sequences.

This invention also provides an antisense oligonucleotide having a sequence capable of binding with mRNAs encoding the enzyme so as to prevent the translation of said mRNA.

This invention also provides transgenic non-human animals comprising a nucleic acid molecule encoding the enzyme. Also provided are methods for use of said transgenic animals as models for mutation and SAR (structure/activity relationship) evaluation as well as in drug screens.

This invention further provides a method of screening compounds to identify those compounds which inhibit the enzyme comprising contacting isolated enzyme with a test compound and measuring the rate of turnover of an enzyme substrate as compared with the rate of turnover in the absence of test compound.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and

secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the sense strand of DNA.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide when placed under the control of appropriate regulatory sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the expression (i.e., the transcription and translation) of a coding sequence in a host cell.

A control sequence "directs the expression" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eukaryotic cells, a stably transformed or transfected cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA or polypeptide sequences are "substantially homologous" or "substantially the same" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule and includes allelic variations. As used herein, substantially homologous also refers to sequences showing identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., "Current Protocols in Mol. Biol." Vol. I & II, Wiley Interscience. Ausbel et al. (ed.) (1992). Protein sequences that are substantially the same can be identified by proteolytic digestion, gel electrophoresis and microsequencing.

The term "functionally equivalent" intends that the amino acid sequence of the subject protein is one that will exhibit enzymatic activity of the same kind as that of Lp-PLA_2.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature.

This invention provides an isolated nucleic acid molecule encoding the enzyme Lp-PLA_2. One means for isolating the coding nucleic acid is to probe a human genomic or cDNA library with a natural or artificially designed probe using art recognized procedures (See for example: "Current Protocols in Molecular Biology", Ausubel, F.M., et al. (eds.) Greene Publishing Assoc. and John Wiley Interscience, NewYork, 1989,1992); for example using the protein fragment information disclosed herein. The enzyme of this invention may be made by recombinant genetic engineering techniques. The isolated nucleic acids particularly the DNAs can be introduced into expression vectors by operatively linking the DNA to the necessary expression control regions (e.g. regulatory regions) required for gene expression. The vectors can be introduced into the appropriate host cells such as prokaryotic (e.g., bacterial), or eukaryotic (e.g. yeast, insect or mammalian) cells by methods well known in the art (Ausubel et al.,supra).The coding sequences for the desired proteins having been prepared or isolated, can be cloned into a suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (E. coli), pBR322 (E. coli), pACYC177 (E. coli),

pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), a baculovirus insect cell system, , YCp19 (Saccharomyces). See, generally, "DNA Cloning": Vols. I & II, Glover et al. ed. IRL Press Oxford (1985) (1987) and; T. Maniatis et al. ("Molecular Cloning" Cold Spring Harbor Laboratory (1982).

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The protein sequences of the present invention can be expressed using, for example, the E. coli tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the coding sequences may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site. Modification of the coding sequences may also be performed to alter codon usage to suit the chosen host cell, for enhanced expression.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the enzyme of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of prokaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,578,355; 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491. pSV2neo (as described in J. Mol. Appl. Genet. 1:327-341) which uses the SV40 late promoter to drive expression in mammalian cells or pCDNA1neo, a vector derived from pCDNA1(Mol. Cell Biol. 7:4125-29) which uses the CMV promoter to drive expression. Both these latter two vectors can be employed for transient or stable(using G418 resistance) expression in mammalian cells. Insect cell expression systems, e.g., Drosophila, are also useful, see for example, PCT applications US 89/05155 and US 91/06838 as well as EP application 88/304093.3.

Depending on the expression system and host selected, the enzyme of the present invention may be produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates or recovered from the cell membrane fraction. Where the protein is localized to the cell surface, whole cells or isolated membranes can be used as an assayable source of the desired gene product. Protein expressed bacterial hosts such as E. coli may require isolation from inclusion bodies and refolding. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The identification of this novel target for the treatment of atherosclerosis, also leads to a novel diagnostic method to diagnose a patient's susceptibility to developing atherosclerotic disease.

The present invention therefore provides in a still further aspect a diagnostic method comprising isolating a sample of blood from the patient, and assaying said

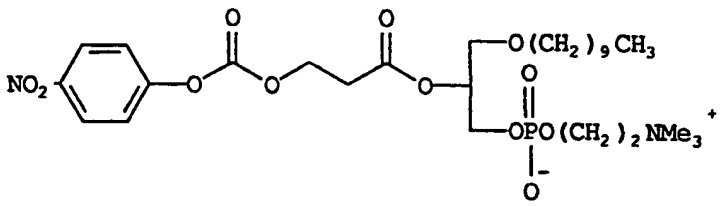
sample for Lp-PLA_2 activity. Patients that are susceptible to atherosclerotic disease are expected to have elevated levels of the Lp-PLA_2 enzyme, and hence the levels of Lp-PLA_2 provides an indication of the patient's susceptibility to atherosclerotic disease. Moreover, Lp-PLA_2 is found located predominantly on dense subfraction(s) of LDL which are known to be very atherogenic. Plasma Lp-PLA_2 levels could therefore provide a ready measure of these very atherogenic small dense LDL particles.

It is expected that the presence of the enzyme in the blood sample of the patient can be assayed by analysis of enzyme activity (i.e. by an assay set up against the purified enzyme as standard); or alternatively by assaying protein content of the sample by using polyclonal or monoclonal antibodies prepared against the purified enzyme. Monoclonal (and polyclonal) antibodies raised against the purified enzyme or fragments thereof are themselves novel and form a further aspect of the invention.

DATA AND EXAMPLES

1. **Screen for Lp-PLA$_2$ inhibition.**

Enzyme activity was determined by measuring the rate of turnover of the artificial substrate (A) at 37 °C in 50mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer containing 150mM NaCl, pH 7.4.



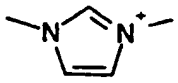
(A)

Assays were performed in 96 well titre plates.

Lp-PLA$_2$ was pre-incubated at 37 °C with vehicle or test compound for 10 min in a total volume of 180 µl. The reaction was then initiated by the addition of 20 µl 10x substrate (A) to give a final substrate concentration of 20 µM. The reaction was followed at 405 nm for 20 minutes using a plate reader with automatic mixing. The rate of reaction was measured as the rate of change of absorbance.

RESULTS:

Compound No	XR^1	R	Z	IC$_{50}$(µM)
1	$O(CH_2)_{15}CH_3$	CH_3CONH	$N^+(CH_3)_3$	0.8
2	$O(CH_2)_{17}CH_3$	CH_3CONH	$N^+(CH_3)_3$	3.5
3	$O(CH_2)_{17}CH_3$	CH_3CONH	$S^+(CH_3)_2$	1.0
4	$O(CH_2)_{17}CH_3$	CH_3CONH	SCH_3	0.08

Compound No	XR^1	R	Z	$IC_{50}(\mu M)$
5	$O(CH_2)_{17}CH_3$	CH_3CONH	OH	0.45
6	$O(CH_2)_{17}CH_3$	CH_3CONH	OAc	0.2
7	$O(CH_2)_{17}CH_3$	CH_3CONH		0.5
8	$O(CH_2)_{17}CH_3$	CH_3CONH		0.55
9	$O(CH_2)_{17}CH_3$	CF_3CONH	$N^+(CH_3)_3$	2.5

2. Copper Stimulated LDL Oxidation:

Copper stimulated oxidation of LDL is routinely measured by following the increase in conjugated diene formation by monitoring the change in absorption at 234 nm. This assay can be used to study inhibitors of oxidative modification of LDL. Figure 1 demonstrates that Lp-PLA_2 inhibitors are effective inhibitors of LDL oxidation through a prolongation of the lag phase, using compound 4 as an example.

3. Inhibition of Cu^{2+} stimulated lyso-phosphatidylcholine (lyso-PtdCho) formation.

A 1 ml aliquot of human LDL (0.25mg protein/ml) was incubated for 15 min at 37 °C with compound or vehicle. 5 µM Cu^{2+} was then added to allow oxidation/lyso-PtdCho formation to occur. The incubation was terminated by the addition of 3.75 ml chloroform/methanol/c HCl (200:400:5,v/v/v). Following the addition of 1.25 ml chloroform and 1.25 ml 0.1M HCl, the mixture was vortexed and centrifuged. The lower phase was carefully removed and the upper phase re-extracted with an equal volume of synthetic lower phase. The extracts were pooled and dried under nitrogen.

Phospholipids were reconstituted into 50 µl chloroform/methanol (2:1 v/v). 10 µl aliquots were spotted on to pre-run silica gel HPTLC plates and then developed in chloroform/methanol 25-30% methylamine (60:20:5 v/v/v). Plates were subsequently sprayed with the flourescent indicator, 2-p-toluidinylnaphthalene-6-sulphonic acid (1 mM in 50 mM Tris/HCl, pH 7.4) to identify phospholipid components. Fluorescence was measured at 222nm using a CAMAG TLC scanner.

Lipoprotein lyso-PtdCho content was quantified using a standard curve (0.05-0.5μg) prepared in parallel.

Compound 4 dose dependently inhibits LDL lyso-PtdCho accumulation stimulated by copper ions with an IC_{50} value of ~30 μM.

4. Purification of lipoprotein associated Lp-PLA_2

Low density lipoprotein (LDL) was obtained by plasma apheresis. The LDL was dialysed against 0.5 M NaCl, 50 mM MES (4-morpholine ethane sulphonic acid), pH = 6.0 overnight at 4°C. Solid CHAPS (3-[(-3-cholamidopropyl)dimethylamino]-1-propane sulphonate) was added to 10 mM and the LDL stirred for 30 minutes to effect solubilisation. The solubilised LDL was pumped onto a pre-equilibrated Blue Sepharose 6FF (Pharmacia) column (2.6 x 20 cm). The column was then washed with 50 mM MES, 10 mM CHAPS, 0.5 M NaCl, pH = 6.0 followed by 50 mM Tris, 10 mM CHAPS, 0.5 M NaCl, pH = 8.0 until the absorbance (280 nm) of the eluate reached zero. Lp-PLA_2 was eluted using 50 mM Tris, 10 mM CHAPS, 1.5 M NaCl, pH = 8.0. The Lp-PLA_2 fraction was then concentrated and dialysed overnight against 50 mM Tris, 10 mM CHAPS, pH = 8.0.

The dialysed Lp-PLA_2 was submitted to anion exchange chromatography on a mono Q column (Pharmacia) using 50 mM Tris, 10 mM CHAPS, pH = 8.0 with a NaCl gradient from zero to 0.3 M. The Lp-PLA_2 fractions obtained from the mono Q column were applied directly to a Hi Trap Blue cartridge (Pharmacia). The cartridge was washed with 50 mM Tris, 10 mM CHAPS, 0.5 M NaCl, pH = 8.0 until the absorbance of the eluate (280 nm) was zero. Lp-PLA_2 was then eluted using 50 mM Tris, 10 mM CHAPS, 1.5 M NaCl, pH = 8.0. This gave Lp-PLA_2 which is greater than 95% pure as shown in Figure 2. This also demonstrates that the native enzyme is extensively glycosylated.

5. Enzyme Sequence

The purity of the final enzyme preparation was verified by five criteria 1) SDS-polyacrylamide gel electrophoresis gave one band for both native and de-glycosylated forms. 2) Reverse phase high pressure liquid chromatography (RP-HPLC) gave a single peak, 3) The intact preparation gave no results by protein sequencing, implying that the protein was N-terminally blocked and free of any contaminants with open N-terminals, 4) By laser desorbtion mass spectometry only one broad peak was observed with de-glycosylated protein, and 5) none of the sections of extended peptide data from sequencing gave any databse matches indicative of contaminating proteins. Three cleavage strategies were used to obtain internal sequence information; trypsin (after de-glycosylation), cyanogen bromide (methionine cleavage) and BNPS-Skatol (tryptophan cleavage). The resulting

peptides were separated by RP-HPLC, collected and sequenced. The accumulated sequence data allowed several extended stretches of primary structure of the Lp-PLA2 enzyme to be verified. These are shown below as Peptides 1, 2, 3 and 4 (SEQ ID Nos 1 to 4). When searched against the National Centre for Biotechnological information (NCBI) non-redundant peptide sequence databases no high similarity matches were obtained. Estimation of the molecular weight of pure, de-glycosylated protein by laser desorption mass spectometry gives values in the region of 45-47kDa (separately 45kDa and 46-47kDa), indicating that the sequences constitute approximately 15 to 20% of the protein.

6. Gene Sequence

Three expressed sequence tags (EST's) from human cDNA libraries have been found to have extensive alignments with the Peptide Sequences 1 to 3. These EST's are shown below as Nucleotide Sequences 1 to 3 (SEQ ID Nos: 5 to 7) Nucleotide Sequence 1 is a 420 base sequence derived from a human foetal spleen library. Nucleotide Sequence 2 is a 379 base sequence derived from a 12-week human embryo library. Nucleotide Sequence 3 is a 279 base sequence derived from a T-cell lymphoma library. The identities at both the nucleic acid and amino acid level justified an overlapping alignment of the cDNA of all three ESTs, Nucleotide Sequences 3 (bases 1-278), 1 (bases 1-389) (in reverse orientation) and 2 (bases 1-304) with the Peptide Sequences 1, 2 and 3 (partially). Beyond these limits, the poor resolution of the raw sequence data precludes accurate base calling.

There are two remaining unassigned peptide sections from Peptides 3 and 4, both of which are expected to be present in the complete protein, -Q-Y-I-N-P-A-V-, and W-L-M-G-N-I-L-R-L-L-F-G-S-M-T-T-P-A-N-.

7. Isolation of full-length Lp-PLA_2 cDNA

The full DNA sequence was determined for the clone (HLTA145) from which the Lymphoma EST (SEQ ID No:7) was derived, giving a total of 572 bases; SEQ ID No:8. There is one base difference between this sequence and the EST (between bases 1 to 256 of the EST); at position 27 of HLTA145 there is an A compared with a T in the EST. This would cause a coding change; L in HLTA145 compared with F in the EST. Clone HLTA145 was used as a radiolabelled probe to screen the Lymphoma cDNA library in order to isolate the full-length Lp-PLA_2 clone. The library was prepared in the bacteriophage λ vector, Unizap XR (Stratagene).

Preparation of the filters for screening

The library was plated out at a density of 20,000 plaques per 150mm petri dish onto *E.coli* XL-1 Blue host cells (ie. 200,000 plaques on 10 dishes). An overnight of XL-1 Blue was prepared in 100mls LB/0.2%w/v Maltose/10mM $MgSO_4$. The cells were pelleted, resuspended in 50mls 10mM $MgSO_4$ and stored on ice. 180µl of the library bacteriophage stock (23,400 pfu's) were added to 7mls XL-1 Blue cells, mixed and divided into 10 aliqouts of 615µl. The 10 tubes were incubated at 37°C for 30 minutes. 7mls of molten (@45°C) top agarose (0.7%w/v agarose in LB) were added, mixed well and poured onto 150mm LB agar plates (1.5%w/v agar in LB). The plates were inverted and incubated at 37°C for approximately 7.5 hours. The plates were held at 4°C until needed.

The plaques were transfered to 132mm Hybond-N nylon filters (Amersham International) by laying the filters on the plates for 2 minutes (4 minutes for the duplicate). The DNA's on the filters were denatured for 2 minutes (0.5M NaCl,1.5M NaOH), neutralised for 4 minutes (1.5M NaCl,1.0M Tris pH7.4) and the filters placed on 2 x SSC for 1 minute. The filters were then dried and the DNA cross-linked to the filter using a Stratalinker UV 2400 (Stratagene) at 120,000 µJoules/cm^2.

The filters were pre-hybridised in 1mM EDTA, 0.5M $NaHPO_4$, 7%SDS (Church,GM. and Gilbert,W. (1984) PNAS USA **81** p1991-1995) in a Techne HB2 hybridisation oven at 55°C for 3 hours. Each bottle contained 2 filters and 25mls prehybridization solution.

Preparation of the radiolabelled probe

The probe cDNA (from HLTA 145) was excised from pBluescript II SK+/- as an approximately 600bp EcoRI-XhoI fragment and approximately 100ng of gel purified fragment were labelled using 1.2MBq ^{32}P dATP and 1.2MBq ^{32}P dCTP by PCR labelling using Taq DNA polymerase (Boehringer Mannheim) and primers designed to prime at the 5' and 3' ends of the EST sequence. The labelling reaction was carried out in a total volume of 200µl and included unlabelled dNTP's at the following concentrations:-

dATP	20µM
dCTP	20µM
dGTP	200µM
dTTP	200µM

The PCR reaction was carried out over 35 cycles of:-

94°C for 30s
60°C for 30s
72°C for 30s

Screening

The radiolabelled probe was denatured at 98°C for 5 minutes and divided into 10 aliquots of 20µl. One aliquot was added per hybridisation bottle. Hybridisation was carried out over 16 hours at 55°C. The filters were washed at 60°C (2 x 10minutes) with 0.1%w/v SDS, 0.1xSSC (50mls per wash per bottle). The filters were autoradiographed and the films (Fuji Medical X-Ray Film) developed after 5 days exposure.

Duplicate positives were progressed to a secondary screen. The plaques were cored out into 1ml SM (100mM NaCl, 10mM $MgSO_4$, 1M Tris, pH7.4), titrated and plated onto 90mm petri dishes at between 20 and 200 pfu's per dish. The secondary screen was carried out as described for the primary screen except the filters were washed at 65°C. The autoradiographs were developed after 16 hours exposure.

DNA sequencing

The duplicated positive clones from the secondary screen were excised from the λ Unizap XR bacteriophage vector into the Bluescript phagemid (according to the Stratagene manual) for characterisation. One of the clones, carrying an insert of approximately 1.5kb, was sequenced on both strands (using the USB Sequenase 2.0 DNA sequencing kit) by primer walking (SEQ ID No:9). The cDNA has an open reading frame with the potential to code for a polypeptide of 441 amino acids.

The 3' region of the full-length cDNA aligns with the HLTA145 sequence with the exception of 3 mismatches (see below). The predicted polypeptide sequence of the lymphoma Lp-PLA_2 is shown as SEQ ID No:9.

Inspection of the full length cDNA (SEQ ID No: 9) reveals probable errors in Peptide 3. One of these errors is in the assignment of continuity between V-M which is incompatible with the perfect sequence match with the cDNA after this position. It seems likely that a short peptide, containing the sequence -Q-Y-I-N-P-, co-purified with the longer cyanogen bromide partial cleavage peptide and, by being present in greater quantity, was assigned as the major sequence and contiguous with the subsequent amino acids. The remaining section of Peptide 3 and the whole of Peptide 4 can be identified in the predicted full length enzyme sequence (SEQ ID No:9). It thus seems likely that Peptide 3 is in fact two separate Peptides 5 (SEQ ID No:10) and 6 (SEQ ID No:11). The second probable error has occurred in the transcription from the raw data for Peptide 3 which on checking was consistent with Peptide 5 having the sequence -Q-Y-I-N-P-V-A, rather than Q-Y-I-N-P-A-V-.

The 3 base differences are as follows:-

1) T at 859 is A in HLTA145; aminoacid change F in full-length, L in HLTA145. (Note that the original EST is identical with the full-length cDNA at position 859).

2) C at 1173 is T in HLTA145; aminoacid change A in full-length, V in HLTA145.

3) T at 1203 is C in HLTA145; aminoacid change L in full-length, S in HLTA145.

The peptide data and the Foetal Spleen EST sequence (SEQ ID No:5) support the full-length cDNA sequence for differences (2) and (3) although the Human Embryo EST (SEQ ID No:6) is identical to the Lymphoma EST (SEQ ID No:7) at position 1173. The Human Embryo EST (SEQ ID No:6) has a further difference (4) corresponding to position 1245 in the full-length Lymphoma sequence (SEQ ID No:9)(comparison between bases 2 to 304 of the Human Embryo EST and the full-length Lymphoma cDNA).

4) A at 1245 is T in the Embryo EST (SEQ ID No:6)(amino acid change D to V in the Embryo EST). Peptide data covering this region supports the Lymphoma DNA sequence (SEQ ID No:9).

The Lp-PLA_2 DNA sequence from 848 to 1361 of SEQ ID No:9 (amino acid residues 271 to 441 of SEQ ID No:9) is the region for which all major data sets agree substantially, ie. the peptide data, the Foetal spleen, full-length Lymphoma and it includes the known active site and is therefore believed to be a significant characterising region for the Lp-PLA_2 enzyme.

The predicted MW for the full reading frame is 50090. This in in exess of that determined for the de-glycosylated, purified protein but post-translational events could account for this discrepancy. The most likely of these are the removal of an N-terminal signal peptide and/or limited proteolytic degradation of the protein C-terminal. The latter could occur in-vivo, during purification, or under the conditions of de-glycosylation.

DIAGNOSTIC METHOD

A sample of blood is taken from a patient, the plasma/serum sample prepared and passed through a dextran sulphate column pre-equilibrated with 0.9% (w\v) NaCl solution. Following washes with the same salt solution Lp-PLA_2 is eluted with a 4.1% (w\v) NaCl solution. Heparin agarose columns can also be used with the wash and elution solutions containing 0.7% and 2.9% NaCl, respectively. Enzyme present in the sample is determined by assaying for either

(a) enzyme activity:

The substrate (A) (see structure in 1) is used to assay Lp-PLA_2 activity by monitoring the absorbance change at 400 nm. Purified enzyme is pre-incubated at

37°C and substrate (50 μM) is added after 5 minutes. The absorbance change at 400 nm is monitored for 20 minutes. This substrate has previously been reported as a substrate for classical calcium-dependent PLA_2s. (Washburn, W.N. and Dennis, E.A., J.Amer Chem.Soc., 1990, 112, 2040-2041); or

(b) protein content

Total protein content (i.e. enzyme content) can be determined using polyclonal antiserum raised against purified human Lp-PLA_2. The antisera recognises both native and glycosylated enzyme as measured by immunoprecipitation of activity and Western Blot analysis.

Polyclonal antiserum was prepared as follows. Immunisation of rabbits involved mixing 0.5 ml of purified human Lp-PLA_2 (=100μg) with an equal volume of Freund's complete adjuvant. The final emulsion was given subcutaneously in 4 X 0.25 ml injections. Boosting using a Freund's incomplete adjuvant\antigen mixture (4 X 0.25 ml subcut.; dosage = 50 μg) took place 4 weeks later. Adequate titre was evident at between 6-8 weeks from initial injection.

IN THE FIGURES:

Fig. 1 is a graph of absorbance at 234 nm against time (min) in a study of inhibition of copper (5μM)- stimulated LDL (150μg/ml) oxidation by compound 4 vs control vehicle.

Fig. 2 is an analysis the purified Lp-PLA_2 material of Example 4 following separation by polyacrylamide gel electrophoresis. Lanes 2, 4 and 6 contain adjacent fractions of purified native Lp-PLA_2. Lanes 1, 3 and 5 are fractions 2, 4 and 6 respectively after N-deglycosylation.

Sequence Data:

SEQ. ID. No: 1 -Peptide 1

-M-L-K-L-K-G-D-I-D-S-N-A-A-I-D-L-S-N-K-A-S-L-A-F-L-Q-K-H-L-G-L-H-K-D-F-D-Q-

SEQ. ID. No: 2 - Peptide 2

-W-M-F-P-L-G-D-E-V-Y-S-R-I-P-Q-P-L-F-F-I-N-S-E-Y-F-Q-Y-P-A-N-

SEQ. ID. No: 3 - Peptide 3

-Q-Y-I-N-P-A-V-M-I-T-I-R-G-S-V-H-Q-N-F-A-D-F-T-F-A-T-G-

SEQ. ID. No: 4 - Peptide 4

-W-L-M-G-N-I-L-R-L-L-F-G-S-M-T-T-P-A-N

SEQ. ID. No: 5 - Nucleotide Sequence 1

```
   1  AAAAAACCTA TTTTAATCCT AATTGTATTT CTCTATTCCT GAAGAGTTCT
  51  GTAACATGAT GTGTTGATTG GTTGTGTTAA TGTTGGTCCC TGGAATAAGA
 101  TTCTCATCAT CTCCTTCAAT CAAGCAGTCC CACTGATCAA AATCTTTATG
 151  AAGTCCTAAA TGCTTTTGTA AGAATGCTAA TGAAGCTTTG TTGCTAAGAT
 201  CAATAGCTGC ATTTGAATCT ATGTCTCCCT TTAATTTGAG CATGTGTCCA
 251  ATTATTTTGC CAGTNGCAAA AGTGAAGTCA GCAAAATTCT GGTGGACTGA
 301  ACCCCTGATT GTAATCATCT TTCTTTCTTT ATCAGGTGAG TAGCATTTTT
 351  TCATTTTTAT GATATTAGCA GGATATTGGA AATATTCAGN GTTGNTAAAA
 401  AGNGGNGGCT GAGGGATTCT
```

SEQ. ID. No: 6 - Nucleotide Sequence 2

```
   1  TGCTAATATC ATAAAAATGA AAAAATGCTA CTCACCTGAT AAAGAAAGAA
  51  AGATGATTAC AATCAGGGGT TCAGTCCACC AGANTTTTGC TGACTTCACT
 101  TTTGCAACTG GCAAAATAAT TGGACACATG CTCAAATTAA AGGGAGACAT
 151  AGATTCAAAT GTAGCTATTG ATCTTAGCAA CAAAGCTTCA TTAGCATTCT
 201  TACAAAAGCA TTTAGGACTT CATAAAGATT TTGTTCAGTG GGACTGCTTG
 251  ATTGAAGGAG ATGATGAGAA TCTTATTCCA GGGACCAACA TTAACACAAC
 301  CAATTCAACA CATCATGTTT ACAGAACTTC TTCCAGGGAA TAGGAGGAAA
 351  TACAATTGGG GTTTAAAATA GGTTTTTTT
```

SEQ. ID. No: 7 - Nucleotide Sequence 3

```
   1  GAAGAATGCA TTAGATTTAA AGTTTGATAT GGAACAACTG AAGGACTCTA
  51  TTGATAGGGA AAAAATAGCA GTAATTGGAC ATTCTTTTGG TGGAGCAACG
 101  GTTATTCAGA CTCTTAGTGA AGATCAGAGA TTCAGATGTG GTATTGCCCT
 151  GGATGCATGG ATGTTTCCAC TGGGTGATGA AGTATATTCC AGAATTCCTC
 201  AGCCCCTCTT TTTTATCAAC TCTGAATATT TCCAATATCC TGCTAATATC
 251  ATAAAANTGG AAAAATGCTA CTCACCTGG
```

Seq.ID.No:8 - DNA sequence of HLTA145

```
        10         20         30         40         50
AAAATAGCAG TAATTGGACA TTCTTTAGGT GGAGCAACGG TTATTCAGAC

        60         70         80         90        100
TCTTAGTGAA GATCAGAGAT TCAGATGTGG TATTGCCCTG GATGCATGGA

       110        120        130        140        150
TGTTTCCACT GGGTGATGAA GTATATTCCA GAATTCCTCA GCCCCTCTTT

       160        170        180        190        200
TTTATCAACT CTGAATATTT CCAATATCCT GCTAATATCA TAAAAATGAA
```

```
       210        220        230        240        250
AAAATGCTAC TCACCTGATA AAGAAAGAAA GATGATTACA ATCAGGGGTT

       260        270        280        290        300
CAGTCCACCA GAATTTTGCT GACTTCACTT TTGCAACTGG CAAAATAATT

       310        320        330        340        350
GGACACATGC TCAAATTAAA GGGAGACATA GATTCAAATG TAGCTATTGA

       360        370        380        390        400
TCTTAGCAAC AAAGCTTCAT CAGCATTCTT ACAAAAGCAT TTAGGACTTC

       410        420        430        440        450
ATAAAGATTT TGATCAGTGG GACTGCTTGA TTGAAGGAGA TGATGAGAAT

       460        470        480        490        500
CTTATTCCAG GGACCAACAT TAACACAACC AATCAACACA TCATGTTACA

       510        520        530        540        550
GAACTCTTCA GGAATAGAGA AATACAATTA GGATTAAAAT AGGTTTTTTA

       560        570
AAAAAAAAAA AAAAAAAACT CG
```

SEQ. ID. No:9 - cDNA Sequence of Lymphoma Lp-PLA$_2$

```
       10         20         30         40         50
TGAGAGACTAAGCTGAAACTGCTGCTCAGCTCCCAAGATGGTGCCACCCA
                                        M  V  P  P  K

       60         70         80         90        100
AATTGCATGTGCTTTTCTGCCTCTGCGGCTGCCTGGCTGTGGTTTATCCT
  L  H  V  L  F  C  L  C  G  C  L  A  V  V  Y  P

      110        120        130        140        150
TTTGACTGGCAATACATAAATCCTGTTGCCCATATGAAATCATCAGCATG
F  D  W  Q  Y  I  N  P  V  A  H  M  K  S  S  A  W

      160        170        180        190        200
GGTCAACAAAATACAAGTACTGATGGCTGCTGCAAGCTTTGGCCAAACTA
 V  N  K  I  Q  V  L  M  A  A  A  S  F  G  Q  T  K

      210        220        230        240        250
AAATCCCCCGGGGAAATGGGCCTTATTCCGTTGGTTGTACAGACTTAATG
  I  P  R  G  N  G  P  Y  S  V  G  C  T  D  L  M

      260        270        280        290        300
TTTGATCACACTAATAAGGGCACCTTCTTGCGTTTATATTATCCATCCCA
F  D  H  T  N  K  G  T  F  L  R  L  Y  Y  P  S  Q

      310        320        330        340        350
AGATAATGATCGCCTTGACACCCTTTGGATCCCAAATAAAGAATATTTTT
 D  N  D  R  L  D  T  L  W  I  P  N  K  E  Y  F  W

      360        370        380        390        400
GGGGTCTTAGCAAATTTCTTGGAACACACTGGCTTATGGGCAACATTTTG
  G  L  S  K  F  L  G  T  H  W  L  M  G  N  I  L
```

```
          410        420        430        440        450
AGGTTACTCTTTGGTTCAATGACAACTCCTGCAAACTGGAATTCCCCTCT
R  L  L  F  G  S  M  T  T  P  A  N  W  N  S  P  L

          460        470        480        490        500
GAGGCCTGGTGAAAAATATCCACTTGTTGTTTTTTCTCATGGTCTTGGGG
 R  P  G  E  K  Y  P  L  V  V  F  S  H  G  L  G  A

          510        520        530        540        550
CATTCAGGACACTTTATTCTGCTATTGGCATTGACCTGGCATCTCATGGG
  F  R  T  L  Y  S  A  I  G  I  D  L  A  S  H  G

          560        570        580        590        600
TTTATAGTTGCTGCTGTAGAACACAGAGATAGATCTGCATCTGCAACTTA
F  I  V  A  A  V  E  H  R  D  R  S  A  S  A  T  Y

          610        620        630        640        650
CTATTTCAAGGACCAATCTGCTGCAGAAATAGGGGACAAGTCTTGGCTCT
 Y  F  K  D  Q  S  A  A  E  I  G  D  K  S  W  L  Y

          660        670        680        690        700
ACCTTAGAACCCTGAAACAAGAGGAGGAGACACATATACGAAATGAGCAG
  L  R  T  L  K  Q  E  E  E  T  H  I  R  N  E  Q

          710        720        730        740        750
GTACGGCAAAGAGCAAAAGAATGTTCCCAAGCTCTCAGTCTGATTCTTGA
V  R  Q  R  A  K  E  C  S  Q  A  L  S  L  I  L  D

          760        770        780        790        800
CATTGATCATGGAAAGCCAGTGAAGAATGCATTAGATTTAAAGTTTGATA
 I  D  H  G  K  P  V  K  N  A  L  D  L  K  F  D  M

          810        820        830        840        850
TGGAACAACTGAAGGACTCTATTGATAGGGAAAAAATAGCAGTAATTGGA
  E  Q  L  K  D  S  I  D  R  E  K  I  A  V  I  G

          860        870        880        890        900
CATTCTTTTGGTGGAGCAACGGTTATTCAGACTCTTAGTGAAGATCAGAG
H  S  F  G  G  A  T  V  I  Q  T  L  S  E  D  Q  R

          910        920        930        940        950
ATTCAGATGTGGTATTGCCCTGGATGCATGGATGTTTCCACTGGGTGATG
 F  R  C  G  I  A  L  D  A  W  M  F  P  L  G  D  E

          960        970        980        990       1000
AAGTATATTCCAGAATTCCTCAGCCCCTCTTTTTTATCAACTCTGAATAT
  V  Y  S  R  I  P  Q  P  L  F  F  I  N  S  E  Y

         1010       1020       1030       1040       1050
TTCCAATATCCTGCTAATATCATAAAAATGAAAAAATGCTACTCACCTGA
F  Q  Y  P  A  N  I  I  K  M  K  K  C  Y  S  P  D

         1060       1070       1080       1090       1100
TAAAGAAAGAAAGATGATTACAATCAGGGGTTCAGTCCACCAGAATTTTG
 K  E  R  K  M  I  T  I  R  G  S  V  H  Q  N  F  A
```

```
      1110       1120       1130       1140       1150
CTGACTTCACTTTTGCAACTGGCAAAATAATTGGACACATGCTCAAATTA
  D  F  T  F  A  T  G  K  I  I  G  H  M  L  K  L

      1160       1170       1180       1190       1200
AAGGGAGACATAGATTCAAATGCAGCTATTGATCTTAGCAACAAAGCTTC
K  G  D  I  D  S  N  A  A  I  D  L  S  N  K  A  S

      1210       1220       1230       1240       1250
ATTAGCATTCTTACAAAAGCATTTAGGACTTCATAAAGATTTTGATCAGT
L  A  F  L  Q  K  H  L  G  L  H  K  D  F  D  Q  W

      1260       1270       1280       1290       1300
GGGACTGCTTGATTGAAGGAGATGATGAGAATCTTATTCCAGGGACCAAC
  D  C  L  I  E  G  D  D  E  N  L  I  P  G  T  N

      1310       1320       1330       1340       1350
ATTAACACAACCAATCAACACATCATGTTACAGAACTCTTCAGGAATAGA
I  N  T  T  N  Q  H  I  M  L  Q  N  S  S  G  I  E

      1360
GAAATACAATT
 K  Y  N  .
```

SEQ. ID. No: 10 -Peptide 5

-Q-Y-I-N-P-V-A-

SEQ. ID. No: 11 -Peptide 6

-M-I-T-I-R-G-S-V-H-Q-N-F-A-D-F-T-F-A-T-G-

CLAIMS:

1. The enzyme Lp-PLA_2 in purified form

2. The enzyme Lp-PLA_2 according to claim 1 characterised by one or more partial peptide sequences selected from SEQ ID NOs:1, 2, 3, 4, 10 and 11 and/or by having a molecular weight of at least 45kDa.

3. The enzyme Lp-PLA_2 according to claim 1 or 2 having a molecular weight of 45kDa.

4. The enzyme Lp-PLA_2 according to claim 1 or 2 having a molecular weight of 45-50kDa.

5. The enzyme Lp-PLA_2 according to claim 4 having a molecular weight of 45-47kDa

6. The enzyme Lp-PLA_2 according to claim 5 having a molecular weight of 46-47kDa

7. The enzyme Lp-PLA_2 according to claim 1 characterised by the partial peptide sequence corresponding to residues 271 to 441 of SEQ ID NO:9.

8. The enzyme Lp-PLA_2 according to claim 1 having the sequence given in SEQ ID NO:9, or an enzyme or fragment thereof having Lp-PLA_2 activity and substantially homologous to SEQ ID NO:9.

9. An enzyme fragment selected from SEQ.ID NOs:1, 2, 3, 4, 10 and 11.

10. An isolated nucleic acid molecule encoding Lp-PLA_2 or an antisense analogue thereof.

11. An isolated nucleic acid molecule encoding the enzyme or fragment of any one of claims 1 to 9 or an antisense analogue thereof.

12. An isolated nucleic acid molecule according to claim 10 comprising the sequence corresponding to:
bases 1-389 of SEQ.ID NO:5;
bases 1-304 of SEQ.ID NO:6;
bases 1-278 of SEQ.ID NO:7; or
SEQ.ID NO:8;
or an antisense analogue thereof.

13. An isolated nucleic acid molecule according to claim 10 comprising the sequence corresponding to bases 848 to 1361 of SEQ ID NO:9 or an antisense analogue thereof.

14. An isolated nucleic acid molecule according to claim 10 consisting of bases 1 to 1361 or 38 to 1361 of SEQ.ID NO:9 or a nucleic acid molecule encoding an enzyme having Lp-PLA_2 activity and substantially homologous to said isolated molecule, or antisense analogues thereof.

15. A recombinant vector comprising the nucleic acid molecule of any one of claims 10 to 14.

16. A host cell comprising the molecule of any one of claims 10 to 14.

17. The use of an inhibitor of the enzyme Lp-PLA_2 in therapy.

18. The use of an inhibitor of Lp-PLA_2 in the treatment of atherosclerosis

19. A method of diagnosis of a patients susceptibility to atherosclerosis which comprises taking a sample of blood from the patient and analysing said sample for the presence of the enzyme Lp-PLA_2.

20. The method according to claim 19 in which the analysis of said sample comprises assaying the sample for enzyme activity.

21. The method according to claim 19 in which the analysis of said sample comprises assaying the sample for protein content using polyclonal or monoclonal antibodies raised against the enzyme.

22. A polyclonal antibody raised against the purified Lp-PLA_2 enzyme as claimed in any of claims 1 to 8.

23. A monoclonal antibody raised against the purified Lp-PLA_2 enzyme as claimed in any of claims 1 to 8.

24. A method of screening compounds to identify those compunds which inhibit the enzyme comprising contacting isolated enzyme Lp-PLA_2 with a test compound and measuring the rate of turnover of an enzyme substrate as compared with the rate of turnover in the absence of test compound.

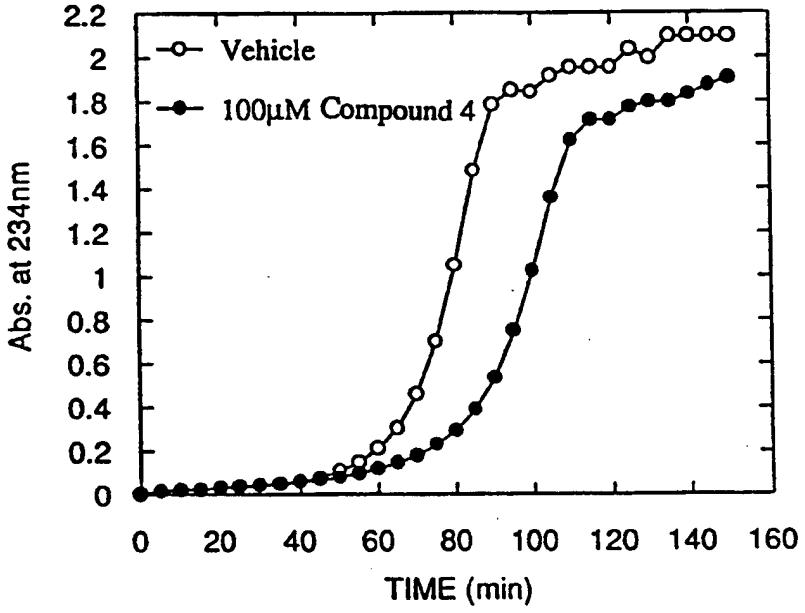


Fig.1 Compound 4 inhibits copper (5µM) - stimulated LDL (150µg/ml) oxidation.

FIGURE 2:

Lanes 2, 4 and 6 contain adjacent fractions of purified native Lp-PLA2. Lanes 1, 3 and 5 are fractions 2, 4 and 6 respectively after N-deglycosylation.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01374

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/55 C12N15/70 C12N9/18 A61K37/64 C12N1/21
C12P21/08 G01N33/573 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K G01N C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 509 719 (ELI LILLY AND COMPANY) 21 October 1992 see page 2, line 26 - line 41 see page 3, line 3 - line 9 see page 5, line 4 - line 13	1,10, 15-17, 22,24
A	WO,A,89 09818 (BIOGEN, INC.) 19 October 1989 see page 8, line 13 - line 24 see page 13, line 30 - page 14, line 25 see page 15, line 8 - page 16, line 5 see page 16, line 19 - line 23	1,10,15, 16,22
A	EP,A,0 359 425 (SHIONOGI & CO. LTD.) 21 March 1990 see page 2, line 46 - page 3, line 13	1,10,15, 16

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1994	18. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/01374

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 17-18 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 94/01374

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0509719	21-10-92	JP-A-	5103677	27-04-93
		US-A-	5328842	12-07-94
WO-A-8909818	19-10-89	AU-A-	3548289	03-11-89
		JP-T-	3503843	29-08-91
EP-A-0359425	21-03-90	JP-A-	2065781	06-03-90
		AU-B-	617209	21-11-91
		AU-A-	4005489	01-03-90